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Identification of malic and soluble oxaloacetate decarboxylase enzymes in *Enterococcus faecalis*

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Two paralogous genes, *maeE* and *citM*, that encode putative malic enzyme family members were identified in the *Enterococcus faecalis* genome. MaeE (41 kDa) and CitM (42 kDa) share a high degree of homology between them (47% identities and 68% conservative substitutions). However, the genetic context of each gene suggested that *maeE* is associated with malate utilization whereas *citM* is linked to the citrate fermentation pathway. In the present work, we focus on the biochemical characterization and physiological contribution of these enzymes in *E. faecalis*. With this aim, the recombinant versions of the two proteins were expressed in *Escherichia coli*, affinity purified and finally their kinetic parameters were determined. This approach allowed us to establish that MaeE is a malate oxidative decarboxylating enzyme and CitM is a soluble oxaloacetate decarboxylase. Moreover, our genetic studies in *E. faecalis* showed that the citrate fermentation phenotype is not affected by *citM* deletion. On the other hand, *maeE* gene disruption resulted in a malate fermentation deficient strain indicating that MaeE is responsible for malate metabolism in *E. faecalis*. Lastly, it was demonstrated that malate fermentation in *E. faecalis* is associated with cytoplasmic and extracellular alkalinization which clearly contributes to pH homeostasis in neutral or mild acidic conditions.

Introduction

Malic enzymes (MEs) catalyse the reversible oxidative decarboxylation of malate to pyruvate and CO_2 with the concomitant reduction of $NAD(P)^+$ to NAD(P)H (Fig. 1A). These enzymes are widely distributed in nature; they have been identified in all life including bacteria, plants and animals [1]. MEs are classified into three groups (EC 1.1.1.38, EC1.1.1.39, EC1.1.1.40) based on their coenzyme requirement and ability to decarboxylate oxaloacetate (OAA) [2]. With regard to prokaryotic MEs, it is worth noting that these proteins are particularly diverse in both size and function and have been less well characterized so far. In *Rhizobium meliloti* two malic enzymes, DME (83 kDa) and TME (82 kDa), have been studied [3]. In *Escherichia coli* an NAD^+- and an $NADP^+$-dependent ME have been identified: ScfA (63 kDa) and MaeB (82 kDa) respectively [4]. Interestingly, in *Bacillus subtilis* four ME isoforms were found, YwkA (64 kDa), MalS (62 kDa), MleA (46 kDa) and YtsJ (43 kDa) [5]. Primary sequence analysis of the aforementioned enzymes reveals that they share a high degree of homology with proteins present in databases which do not show ME activity. Instead, they have been proved to act as malolactic enzymes (MLEs), which catalyse the specific

Abbreviations
LAB, lactic acid bacteria; ME, malic enzyme; MEF, malic enzyme family; MLE, malolactic enzyme; OAA, oxaloacetate; OAD, OAA decarboxylase.

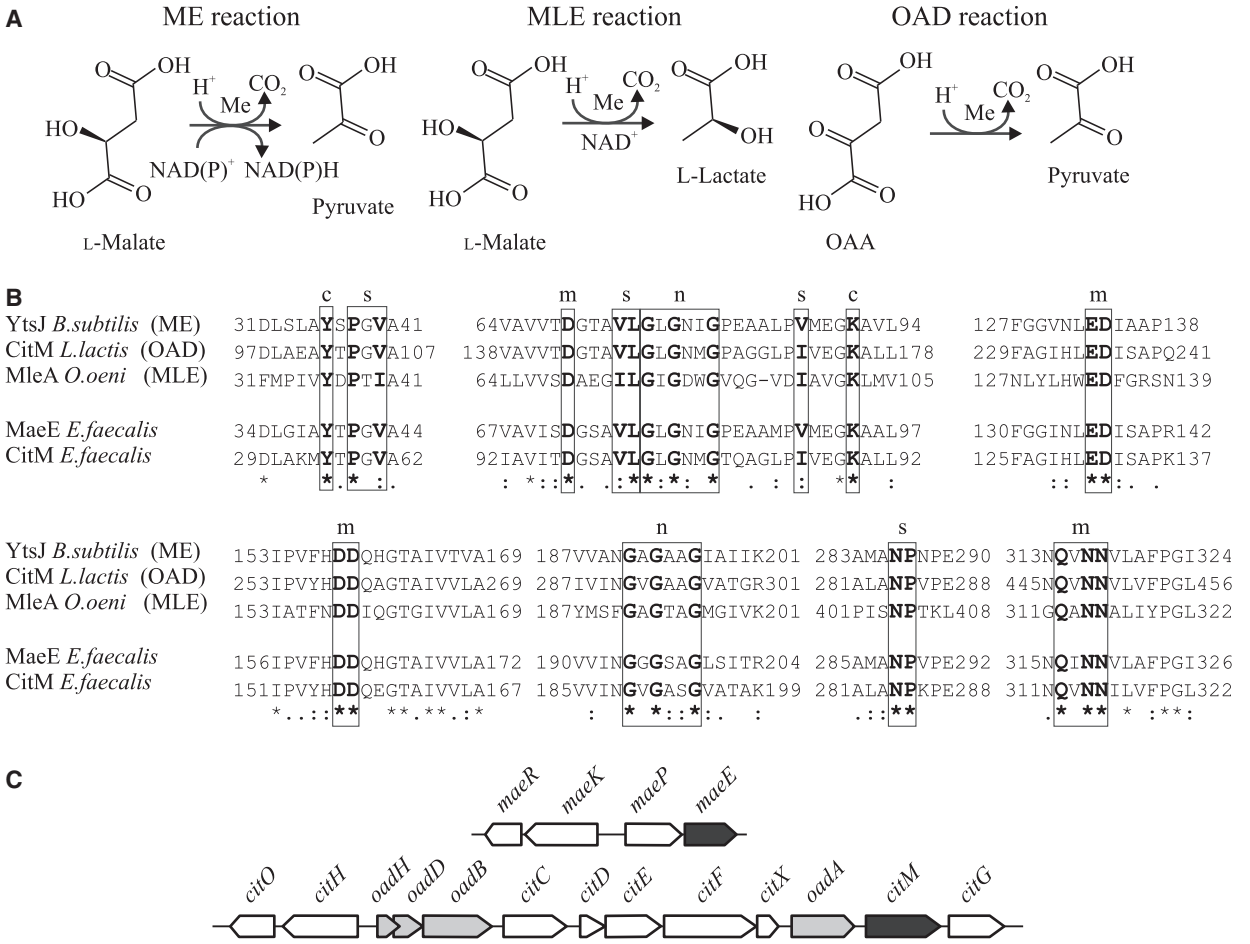

Fig. 1. (A) Reactions catalysed by MEF proteins. ME and MLE are involved in the conversion of L-malate into pyruvate and L-lactate, respectively. OAD enzymes catalyse the decarboxylation of OAA to give pyruvate. The presence of a divalent cation (Me) is required in all cases. (B) Multiple sequence alignments of YtsJ (*B. subtilis*), CitM (*L. lactis*), MleA (*O. oeni*), MaeE and CitM (*E. faecalis*) proteins. Only protein regions with conserved amino acids are shown. Conserved residues implicated in catalysis (c) or substrate (s), divalent cation (m) or NAD(P)$^+$ (n) binding are indicated in boldface. See Table S1 for accession numbers and further details of MEF members included in the alignment. (C) Genetic organization of the *mae* and *cit* locus. Genes coding for MEF proteins are indicated in dark grey while those encoding the membrane bound OAD are shown in grey. See text for details.

decarboxylation of malate to lactate [6] or soluble OAA decarboxylases (OAD), which convert OAA to pyruvate [7] (Fig. 1A). Remarkably, MLE and OAD proteins contain the same conserved amino acids found in the active site of previously characterized MEs, including the catalytic tyrosine and lysine residues involved in the acid–base mechanism, the divalent cation-binding residues, and two Rossman domains (GXGXXG) implicated in cofactor binding [1] (Fig. 1B). For this reason, in this study we refer to MEs, MLEs and OAD enzymes as members of the malic enzyme family (MEF).

MLEs are mainly found in the *Firmicutes* phylum and were initially studied in *Oenococcus oeni* due to the importance of the malolactic fermentation in wine deacidification [6]. This pathway was also characterized in *Lactococcus lactis* and *Streptococcus mutans* where it is involved in metabolic energy generation and survival at low pH [8,9]. Another pathway associated with proton motive force generation in bacteria is citrate fermentation [8,9]. Soluble OADs are specifically involved in this metabolism, with *L. lactis* CitM as the first enzyme to be characterized. This enzymatic reaction converts OAA (derived from citrate) into pyruvate in the presence of divalent metals and in the absence of nicotinamide cofactors [7]. Noteworthy, the activity of MEF proteins contribute to the intracellular pH homeostasis since scalar protons are consumed during the decarboxylative step. Moreover, the external alkalinization of the medium is a well documented

phenotype associated with decarboxylative reactions, which also results in a growth advantage for the cell [8,10].

Enterococcus faecalis is a Gram-positive lactic acid bacterium (LAB) commonly found in the gastrointestinal tract of humans and animals and also present in fermented foods such as cheese, yogurt and sausages. Indeed, some enterococci strains have been used as probiotics [11]. On the other hand, some species of this genus have emerged as important opportunistic antibiotic-resistant pathogens in hospital infections in the last decades [12]. *E. faecalis*, like other LAB members, lacks an active Krebs cycle and several respiratory electron chain proteins. Consequently, it depends mainly on substrate level phosphorylation for energy production. The capability of *E. faecalis* to grow, resist and persist in widely different environmental conditions is based on the variety of transporters and enzymes involved in the metabolism of organic compounds, such as malate and citrate, encoded within its genome [13].

In this work we identified two putative members of the MEF in the *E. faecalis* genome, MaeE and CitM. Biochemical studies confirmed that MaeE is a malate oxidative decarboxylase and CitM is a soluble OAD. Interestingly, after inactivation of *citM* growth parameters of cells cultured in citrate-containing media were not altered, whereas disruption of *maeE* produced a malate-defective phenotype. Finally, we found that MaeE activity provokes cytoplasm and extracellular media alkalinization favouring bacterial growth in mild acidic environments.

Results

Phylogenetic and gene context analysis of the MEF members encoded in *E. faecalis* genome

A sequence analysis of the *E. faecalis* V583 genome revealed the presence of two genes coding for MEF members, *maeE* (EF1206) and *citM* (EF3316). As shown in Fig. 1B, both gene products also contain the conserved residues characteristic of this protein family. MaeE from *E. faecalis* shared 53% with YtsJ from *B. subtilis* [5] and 99% identity with MaeE from *Streptococcus bovis* [14]. In *E. faecalis, maeE* is situated in a locus composed of two putatively divergent operons, *maePE* and *maeKR* (Fig. 1C). *maeE* is located downstream of *maeP*, which codes for a putative H^+/malate symporter belonging to the 2-hydroxycarboxylate family [15]. The other bicistronic operon is formed by the *maeK* (EF1205) and *maeR* (EF1204) genes, which are close homologues of previously described two-component systems involved in sensing citrate or malate in *Es. coli* [16], *B. subtilis* [17] and *Lactobacillus casei* [18]. A subsequent phylogenetic analysis showed that MaeE clusters together with its orthologue from *L. casei* [18] and other putative MEs from closely related LAB. Furthermore, all cluster members share a similar genetic arrangement associated with malate metabolism (Fig. 2A, ME dashed circle).

On the other hand, the *citM* gene is located in the *cit* locus, which is composed of two divergent operons, *citHO* and *oadHDB-citCDEFX-oadA-citMG* (Fig. 1C). *citH* codes for a citrate transporter of the CitMHS family (TC 2.A.11) [19] and *citO* encodes a GntR-like transcriptional regulator. The *oadHDB-citCDEFX-oadA-citMG* operon encodes the catabolic enzymes of the pathway: the citrate lyase and its accessory proteins as well as two putative OADs [20]. One of them is encoded by the *oad* genes and is a homologue of the OAD membrane bound complex from *Klebsiella pneumoniae* [21]. The other is coded by the *citM* gene and has a 55% homology with the soluble decarboxylase characterized in *L. lactis* [7]. *E. faecalis* and *L. lactis* CitMs are together in a specific minor branch of the MEF tree, which is composed of other putative MEF members encoded in each case by genes associated with a cluster of citrate pathway genes (Fig. 2, OAD dashed circle). The presence of two different classes of OADs (*citM* and *oad* genes) in the *E. faecalis* genome is a unique feature among all citrate clusters identified by nucleotide sequence analysis. We found that 23 out of 24 recently assembled genomes, corresponding to diverse *E. faecalis* isolates, contain *citM* as well as *oad* genes. The exception is *E. faecalis* Merz96 strain, which carries a disrupting insertion in *citM*.

Cloning, heterologous expression and characterization of CitM and MaeE from *E. faecalis*

Initially, *citM* and *maeE* genes were amplified using specific primers and DNA extracted from *E. faecalis* JH2-2 as template. The amplimers were further cloned into a pET28a vector, yielding plasmids pET-CitM and pET-MaeE, respectively. Next, *Es. coli* BL21 (DE3) strain was used for the isopropyl thio-β-D-galactoside (IPTG) induced overexpression of the recombinant His6-CitM and His6-MaeE proteins. Finally, both enzymes were purified to homogeneity from the host cell extracts by Ni^{2+}-bounded affinity columns (Fig. 3A; see Materials and Methods for details). To determine whether these recombinant proteins showed malic activity we performed native polyacrylamide gel zymograms. As shown in Fig. 3B, malic activity was detected for purified MaeE but not in the case of CitM

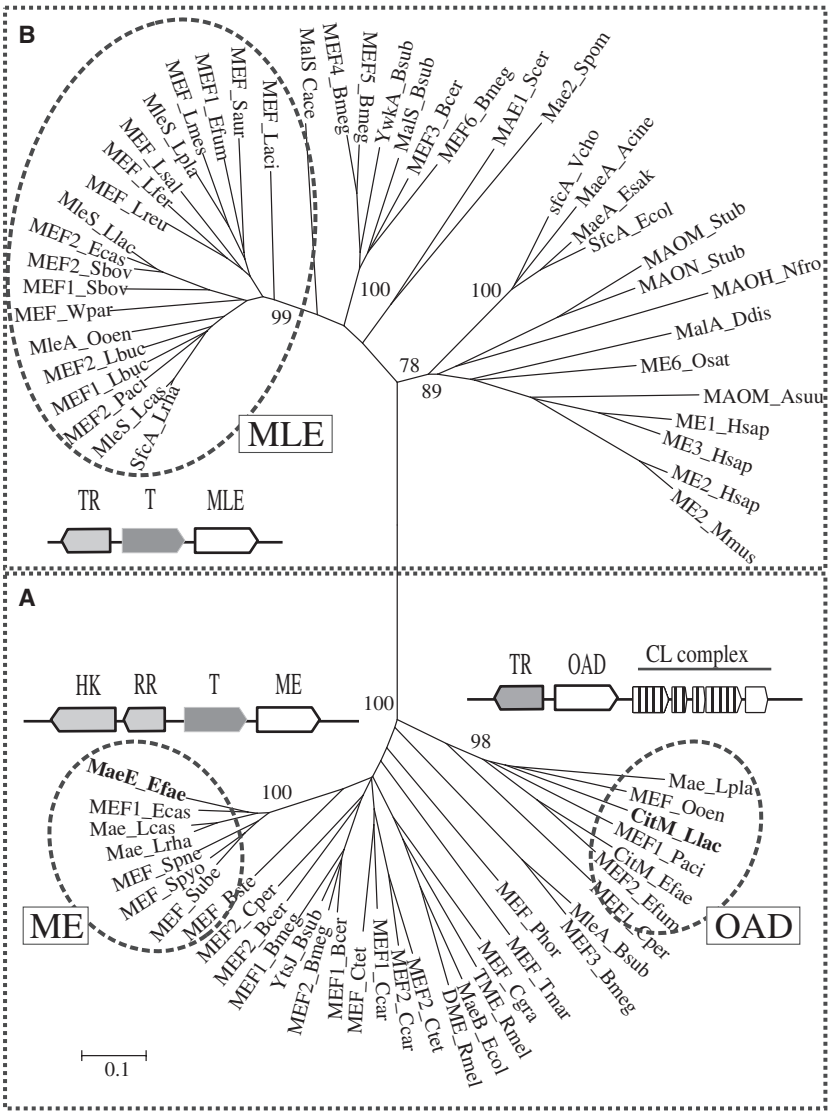

Fig. 2. Unrooted phylogenetic tree constituted by 75 MEF members from various origins (see Table S1 for details). ME, MLE and OAD from LAB are highlighted with dashed circles and main branches A and B are depicted as dashed rectangles. Bootstrap support values of main and minor branches are indicated. Genetic contexts of MEF coding genes from LAB are also indicated. HK, histidine kinase; RR, response regulator; T, transporter; TR, transcriptional regulator; CL, citrate lyase.

(lane 2 and lane 1, respectively). Hence, we decided to evaluate the activities of these enzymes through a complementation test employing the *Es. coli* mutant EJ1321 [22]. This strain is deficient in malic and PEP-carboxykinase activities making it unable to use C4 compounds such as OAA, succinate or malate as a carbon source since it cannot convert them into C3 compounds. Therefore, the EJ1321 strain harbouring pREP4 was co-transformed with pQE30-plasmid derivatives carrying a copy of *citM* or *maeE* (pQE-CitM or pQE-MaeE, respectively; see Materials and Methods for details). The *Es. coli* defective strain transformed with the pQE30 empty vector showed a limited growth in MSMYE medium [35] supplemented with succinate (Fig. 3C). Conversely, *maeE*- and *citM*-expressing strains reached higher biomass levels (Fig. 3C), suggesting that the corresponding gene products were complementing the deficient strain. In these two strains succinate is converted into fumarate and then oxidized to malate. The latter is further decarboxylated to pyruvate by the action of MaeE which allows strain growth. On the other hand, for the *citM*-complemented strain, malate could be first converted into OAA by the endogenous malate dehydrogenase enzyme and then decarboxylated to pyruvate by CitM.

To confirm this hypothesis, we analysed the enzymatic activities of both enzymes by *in vitro* biochemical assays. Initially, it was determined that the optimum pH value for MaeE malic activity was 8.5 (not shown). This condition was used to assay the kinetic parameters employing NAD^+ as a cofactor. The $K_{m,malate}$ and k_{cat} for MaeE malic activity were 0.50 ± 0.08 mM and $21.8 \pm 3.8\ s^{-1}$, respectively. Despite small differences in optimum pH (8.5 rather than 7.8), similar kinetic

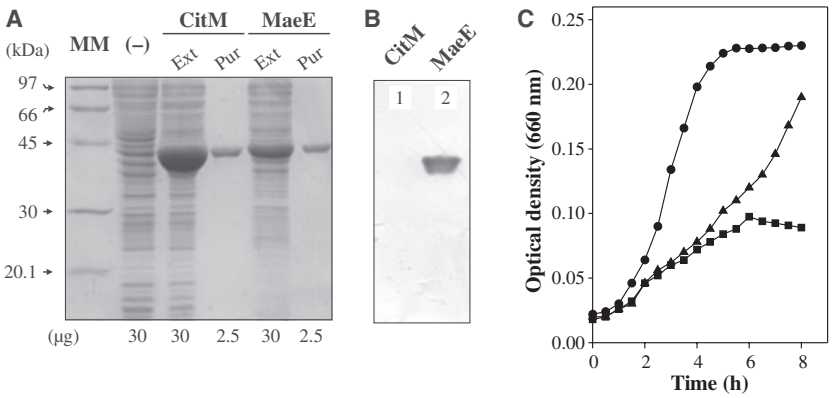

Fig. 3. (A) Coomassie-stained SDS/PAGE of recombinant CitM and MaeE. Soluble cell extracts of IPTG-induced *E. coli* BL21 (DE3) carrying pET28a [(–)], pET-CitM (CitM) or pET-MaeE (MaeE) plasmids were loaded onto the gel, before (Ext for extract) and after (Pur for purified) Ni^{2+}-affinity column purification. MM, molecular mass standard markers. (B) Zymograms for malic activity. 10 μg of each purified recombinant CitM and MaeE proteins (lane 1 and 2, respectively) were loaded onto a polyacrylamide non-denaturing gel and malic activity was developed *in situ*. (C) Growth curves of *E. coli* EJ1321 pREP4 transformed with pQE30 (■), pQE-CitM (▲) or pQE-MaeE (●) plasmid. Cells were grown in MSMYE medium supplemented with 80 mM succinate.

constants were obtained for the *S. bovis* ME [14]. In contrast to the observations reported for its orthologue from *S. bovis*, we were able to measure *E. faecalis* MaeE OAD activity when the assays were performed in the pH range between 4.5 and 5.5, with an optimum value at 5.0. Hence, kinetic constants for this activity were determined at this pH resulting in a $K_{m,OAA}$ of 0.59 ± 0.20 mM and k_{cat} of 206.7 ± 23.3 s^{-1}. Surprisingly, MaeE showed a higher catalytic efficiency for the OAA to pyruvate conversion ($k_{cat}/K_{m,OAA}$ 365.0 ± 81.7 $mM^{-1}{\cdot}s^{-1}$) than for the malate to pyruvate reaction ($k_{cat}/K_{m,malate}$ 43.3 ± 1.0 $mM^{-1}{\cdot}s^{-1}$).

Next, the OAD activity of purified CitM was assayed in the 3.5–5.0 pH range, observing an optimum pH value of 4.5 (data not shown). Thus, we calculated the kinetic parameters at this pH. $K_{m,OAA}$, k_{cat} and $k_{cat}/K_{m,OAA}$ were 0.62 ± 0.31 mM, 11.2 ± 3.3 s^{-1} and 22.3 ± 16.6 $mM^{-1}{\cdot}s^{-1}$, respectively. OAD activity was dependent on the presence of divalent metal ions and inhibited in the presence of 2 mM EDTA (not shown). Although CitM has all the conserved residues of MEF members (Fig. 1B), no malic activity could be detected under any tested condition. These results are similar to those previously reported for its orthologue from *L. lactis* [7].

Effect of different metabolites and metals on MaeE and CitM activities

The nature of the effectors that modulate the activity of an enzyme can usually provide some clues about its actual physiological role. MEs from plants, animals and some bacteria have been shown to be highly allosterically regulated [1,4,23]. For this reason, we explored the effect of the addition of different key metabolites on MaeE and CitM activities. In particular, we scrutinized the effect of citrate, key intermediates (pyruvate, acetyl-CoA, acetyl phosphate and CoA) and major end products (acetate and lactate) of citrate and malate metabolism. These assays indicated that citrate exerted a moderate inhibition on both enzymes with a more pronounced effect on MaeE malic activity (Table 1). All other tested metabolites caused no significant variations in malic and OAD activities (not shown). It was previously suggested that *Es. coli* ME may be involved in amino acid and/or lipid biosynthesis. Bearing that in mind, we examined whether aspartate, glutamate or stearyl-CoA could affect MaeE and CitM activities. Inhibition was only observed for MaeE malic activity in the presence of 50 μM stearyl-CoA (Table 1). This effect could not be measured for OAD activity due to low stearyl-CoA solubility in the reaction buffer. Accordingly, *Es. coli* ScfA was inhibited by long chain acyl-CoAs [4].

It was formerly reported by our group that the OAD activity of CitM from *L. lactis* was inhibited by NAD^+ and NADH [7]. These results prompted us to assay the OAD activity of *E. faecalis* MEF enzymes in the presence of the two compounds. Interestingly, the presence of NAD^+ and NADH caused inhibition of CitM OAD activity but not of MaeE (Table 1). Moreover, we assayed the effect of ATP and ADP on the activity of these enzymes since it has been reported that ATP can inhibit human m-NAD-ME by interacting with its conserved NAD^+ binding site [1]. Comparable inhibition was also reported for other bacterial MEs or partially purified MEs from *E. faecalis* [14,24–

Table 1. Effects of diverse metabolites on malic and OAA activities. Malic or OAD activities were measured under standard assay conditions with 0.3 mM malate or OAA as substrates, respectively. Results are presented as the enzyme activity ratio in the presence and absence of compounds. The data correspond to mean values ± SD of at least two independent experiments. For improved reproducibility of OAD activity in (a) the enzymes were pre-incubated with ATP, ADP, NAD or NADH. No malic activity could be measured for CitM. ND, not determined. NT, could not be tested.

	% malic activity	% OAD activity	
	MaeE	CitM	MaeE
2 mM malate		65 ± 5	56 ± 4
2 mM citrate	47 ± 4	74 ± 5	75 ± 6
10 μM stearyl-CoA	75 ± 2	NT	NT
50 μM stearyl-CoA	40 ± 3	NT	NT
0.25 mM NAD^{+}[a]		40 ± 10	104 ± 7
0.25 mM NADH[a]		53 ± 2	89 ± 3
1 mM ATP	5 ± 1	ND	ND
0.25 mM ATP[a]	ND	37 ± 3	12 ± 3
1 mM ADP	41 ± 1	ND	ND
0.25 mM ADP[a]	ND	78 ± 2	71 ± 5
2 mM malonate	49 ± 4	26 ± 6	17 ± 1
2 mM oxalate	< 5 ± 1	14 ± 6	22 ± 1
2 mM oxalacetate	< 5 ± 2		
2 mM succinate	82 ± 1	103 ± 5	ND

28]. Our studies showed that ATP and ADP also inhibited MaeE malic and MaeE and CitM OAD activities. The inhibitory effect of ATP was greater than that exerted by ADP (Table 1).

The consequences of the addition of substrate analogues on CitM and MaeE catalysed reactions were also studied. Both malate and OAA inhibited the OAD and malic activities, respectively (Table 1). Moreover, malonate and oxalate inhibited both enzymes (Table 1) whereas no significant effect was observed for tartrate (not shown). Finally, succinate only mildly inhibited MaeE activity (Table 1).

When metal requirement was analysed, MaeE showed a maximal malic activity at 0.1 mM Mn^{2+} whereas the CitM and MaeE OAD activities required a metal concentration of 20 mM (Table 2). These findings indicate the existence of distinct metal requirements depending on the type of activity measured. Furthermore, both MaeE and CitM were inhibited by the addition of EDTA to the reaction medium highlighting the essential role of divalent metal ion in catalysis (Table 2).

CitM is not required for efficient citrate utilization in *E. faecalis* JH2-2

To determine the CitM contribution to citrate utilization in *E. faecalis*, a *citM* deficient strain was employed. In this strain, a deletion in the central region of the *citM* gene was generated using the chimeric vector pBVGh as described by Blancato and Magni [29]. It is important to note that the construction does not alter the expression of genes downstream of *citM*. Growth curves for *E. faecalis* JH2-2 and *citM* defective strains were then performed. Both strains showed the same growth pattern and reached comparable final biomass levels in LB medium supplemented with 0.5% citrate (not shown). We additionally determined the growth parameters of both strains in different media and under various growth conditions. In order to achieve this, *E. faecalis* strains were grown in LB, M17 and Milk medium [36] containing various citrate concentrations (0–1%). We reduced the initial external pH (pH_i) from 7.0 to 5.0, changed the aeration conditions (static or shaking) and finally we modified the external concentrations of Na^+ (0–500 mM), Mn^{2+} (0–1 mM), EDTA (0–4 mM), aspartate (0–20 mM) and glucose (0–1%). In all cases, we were unable to detect any difference in growth parameters between the *citM* mutant and its parental strain (data not shown). These results show that the *citM* deletion does not cause any modification in growth parameters during citrate fermentation under our experimental conditions.

Table 2. Effect of Mn^{2+} and EDTA on malic and OAD activities. Malic or OAD activities were measured under standard assay conditions with 1.5 mM malate or 1.0 mM OAA as substrate, respectively. The results are presented as the percentage of enzyme activity, in the presence of the indicated $MnCl_2$ concentration and 2 mM EDTA when indicated, in relation to the highest activity measured. The data correspond to mean values ± SD of at least two independent experiments. ND, not determined.

	% malic activity	% OAD activity	
	MaeE	CitM	MaeE
Added Mn^{2+}			
0 mM	81 ± 7	15 ± 3	10 ± 4
0.05 mM	99 ± 4	ND	ND
0.1 mM	100 ± 7	23 ± 10	10 ± 3
0.5 mM	97 ± 7	41 ± 18	33 ± 9
2 mM	83 ± 6	77 ± 13	62 ± 2
20 mM	31 ± 4	100 ± 28	100 ± 2
0.1 mM + EDTA	< 5	ND	ND
0.5 mM + EDTA	ND	< 5	8 ± 1

MaeE is an essential enzyme for malate utilization in *E. faecalis* and contributes to pH homeostasis

To test whether MaeE was required for malate metabolism in *E. faecalis*, we disrupted its coding gene by single crossover chromosomal integration of plasmid pGh9-L. The insertion does not modify the expression

Table 3. Final growth parameters of *E. faecalis* strains cultivated in LB basal medium alone or with the addition of malate adjusted at different initial pHs. *E. faecalis* JH2-2 (wild-type) and its *maeE* derivative mutant were grown without shaking at 37 °C in LB basal medium or LB supplemented with 35 mM malate (LBM). Final A_{660}, extracellular pH (e-pH_f) and residual malate concentration (% of initial concentration ± SD) were determined after 6- and 24-h growth for the corresponding media adjusted at pH_i of 7.0 and 5.5, respectively. The data correspond to a representative experiment of at least three independent assays. ND, not determined.

	pH_i 7.0				pH_i 5.5			
	Wild-type strain		*maeE* strain		Wild-type strain		*maeE* strain	
	LB	LBM	LB	LBM	LB	LBM	LB	LBM
A_{660}	0.48	0.76	0.50	0.51	0.40	0.56	0.29	0.27
e-pH_f	6.5	6.8	6.5	6.5	5.4	6.5	5.3	5.3
Malate (%)	ND	< 2	ND	106 ± 11	ND	< 2	ND	106 ± 5

of the genes coding for the malate transporter (*maeP*) or those encoding for the two-component system involved in *mae* locus regulation (*maeK* and *maeR*) (Fig. 1C). Next, we analysed the growth profile of the *maeE* disrupted mutant and its parental strain in LB basal medium with or without the addition of 35 mM malate (LBM) and adjusted at different pH_i values (7.0, 5.5 and 4.5). As shown in Table 3, when medium pH_i was adjusted to 5.5, a general decrease in final biomass with respect to cells cultured at pH_i 7.0 was detected. Moreover, strains were unable to grow in LB or LBM at pH_i 4.5. The acidic initial conditions also affected growth rate (not shown). Therefore, final parameters of pH_i 5.5 cultures were determined after 24 h instead of the 6 h incubation employed for cultures grown at pH_i 7.0. When the wild-type strain was grown in LBM at pH_i 7.0 or 5.5 it showed an increase in its biomass with respect to LB cultured cells of 58% or 40%, respectively. This growth enhancement was not observed for the *maeE* disrupted strain. In agreement, extracellular malate concentration was exhausted for wild-type cultures grown at pH_i 7.0 or 5.5 for 6 or 24 h, respectively (Table 3). For the wild-type strain malate consumption was followed by an increase in extracellular pH, which was not observed for the same strain grown on LB. In contrast, the *maeE* deficient strain was unable to degrade malate and the concomitant alkalinization of external medium was not detected. The effect of supernatant alkalinization was more evident when the wild-type strain was grown at an external pH_i of 5.5 ($\Delta pH = 1$, Table 3).

To evaluate the contribution of malate metabolism to pH homeostasis, cytoplasmic H^+ levels were monitored by using the pH-sensitive fluorescent probe CDCFD (see Materials and Methods for details). In order to suppress gene induction variation among different growth conditions, both strains were first cultivated in LBM adjusted to pH_i 7.0, loaded with the fluorescent probe and finally equilibrated in resting medium buffered at pH 7.0, 5.5 or 4.5. As shown in Fig. 4, cytoplasmic pH values were higher when wild-type cells were grown at extracellular pH values of 7.0 or 5.5 upon addition of 10 mM malate. At external pH values of 4.5 this strain showed a minor response. Remarkably, no alkalinization was observed for the *maeE* deficient strain at all tested pH values. In sum, these results indicate that MaeE mediates malate utilization in *E. faecalis* and that its malic decarboxylative activity contributes to pH homeostasis during growth in neutral or mild acidic environments.

Discussion

In the present work, we identified two members of MEF proteins in the *E. faecalis* genome encoded by *citM* and *maeE* genes. Characterization of their purified products allowed us to conclude that CitM is an OAD specifically associated with citrate metabolism whereas MaeE is a malate oxidative decarboxylase. Our biochemical studies showed that MaeE malic activity has a requirement for Mn^{2+} of the order of 0.1 mM. However, for MaeE or CitM OAD activities the amount of divalent metal ion needed for catalysis had to be increased 200 times (20 mM). Since CitM from *L. lactis* showed activity in the absence of Mn^{2+} when Mg^{2+} was added to the reaction buffer [7], we hypothesize that Mg^{2+} rather than Mn^{2+} is the physiological metal ion involved in the catalysis of OAD enzymes. Accordingly, it has been shown that bacterial Mn^{2+} and Mg^{2+} content are in the micromolar and millimolar ranges, respectively [30]. The removal of metals by EDTA (2 mM) produced a rapid precipitation of CitM, which was particularly sensitive to the presence of the chelator (Table 2). These results indicate that the presence of a metal ion is not only necessary for the catalytic mechanism but may also be essential for enzyme stability.

Analysis of the effect of substrate analogues on CitM and MaeE OAD activity showed a similar

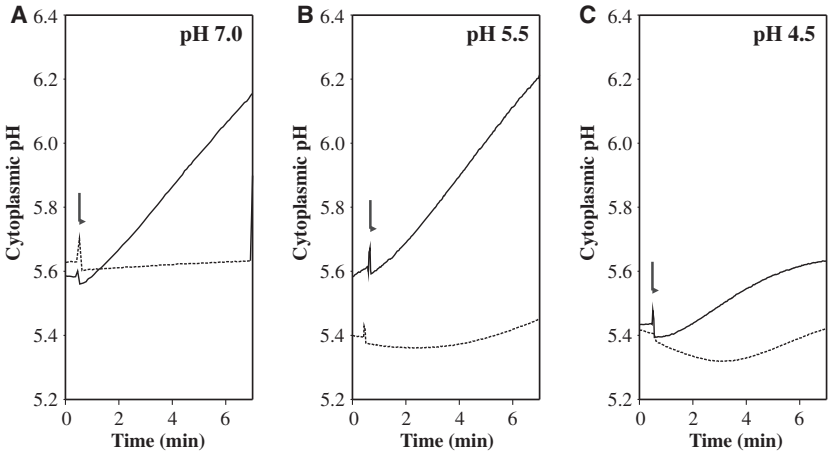

Fig. 4. Role of MaeE in *E. faecalis* cytoplasmic alkalinization associated with malate metabolism. Cytoplasmic pH value variations of wild-type (solid lines) and *maeE* mutant strain (dashed lines) were monitored employing the CDCFD fluorescent probe. Resting cells were suspended in buffer phosphate at pH_i 7.0 (A), 5.5 (B) or 4.5 (C). A pulse of 10 mM malate was added at the time indicated by the arrow. Experiments were performed in triplicate and one representative assay is presented.

degree of inhibition by oxalate, malonate and malate (Table 1). This inhibition could be due to metal sequestration by complex formation with the carboxylic group of these metabolites. However, tartrate, which does not inhibit OAD activity (not shown), has an equilibrium binding constant for complex formation with Mn^{2+} higher than malate and malonate [30]. This suggests that, at least for these two compounds, metal complexation is not the main inhibitory mechanism. Based on structural similarities, it can be inferred that these metabolites may act as competitive inhibitors. Nevertheless, the existence of allosteric regulation could not be ruled out. A striking characteristic of CitM is that it does not catalyse the oxidation of malate to OAA while it is able to decarboxylate the latter to pyruvate. Moreover, our assays showed that NAD^+ or NADH, rather than being required for CitM catalysis, act as enzymatic inhibitors (Table 1). This effect was also described for CitM from *L. lactis* [7] suggesting that such regulation might be relevant *in vivo*. Interestingly, we also observed CitM and MaeE OAD activity inhibition by ATP or ADP (Table 1). We hypothesized that conserved nucleotide binding residues in the active site (Fig. 1B) are presumably involved in the ATP, ADP, NAD and NADH inhibition pattern of CitM and MaeE. However, binding of such compounds to sites distant from the catalytic region or even to different sites could not be excluded by our results. Nevertheless, if our proposition is correct, the absence of malic activity for CitM may be a result of an improper orientation of the cofactor that impairs catalysis or malate binding, as was described for site-directed mutants of *Ascaris suum* NAD-ME and for ATP binding to human m-NAD-ME [31,32]. More detailed work should be conducted to elucidate the current action mechanisms elicited by substrate analogues and purine nucleotide derived compounds on *E. faecalis* MEF proteins.

One of our objectives in this work was to analyse the role that CitM plays in the citrate utilization phenotype of *E. faecalis*. Our genetic studies have shown that a *citM* deletion did not impair citrate metabolism. This result suggests that CitM is either not capable of providing an obvious fitness advantage during citrate fermentation or that the OAD membrane complex could efficiently suppress the CitM deficiency under our experimental conditions. In principle, the MaeE OAD activity could also compensate such deficiency. However, as *maeE* gene is not transcribed in LB basal or citrate supplemented media (our unpublished results) the contribution of MaeE to citrate metabolism should be negligible.

To analyse the physiological role of MaeE in *E. faecalis*, in the present study we analysed final growth parameters and malate consumption profiles of *E. faecalis maeE* defective mutant and its wild-type parental strain. We unambiguously demonstrated that MaeE is essential for malate utilization in *E. faecalis* JH2-2. Cytoplasmic pH values of resting cells resuspended at extracellular pH values of 7.0 or 5.5 were also monitored. Irrespective of external pH values, we observed that cytoplasmic pH was maintained around 5.6. However, upon malate addition cytoplasmic pH increased only when a wild-type copy of *maeE* was present (Fig. 3). This internal alkalinization correlates with the increase of external pH during batch malate fermentations (Table 3). Surprisingly, *E. faecalis* growth was impaired when external pH_i was set to a value of 4.5. Although our results indicate that malate fermentation could contribute to pH homeostasis in mild and neutral environments more acidic conditions seem to be detrimental to *E. faecalis*.

Our phylogenetic analysis showed that MEF proteins clustered in two main branches, named A and B

(Fig. 2). Interestingly, *E. faecalis* CitM and MaeE cluster in branch A suggesting that they share a common phylogenetic origin and presumably they have emerged by duplication of an ancestral gene. On the other hand, MLEs seem to have evolved from a more distant ancestor than ME and OAD from LAB since they clustered in a different branch of the phylogenetic tree (Fig. 2). Remarkably, MLEs are the most widely distributed MEF members among LAB. This is presumably a consequence of their contribution to low pH tolerance [8,9]. The presence of an ME rather than an MLE seems to be restricted to a small group of LAB, including *E. faecalis*. This variability in MEF protein contents among LAB might explain the differences in their observed tolerance to acid milieu [33]. The selection of an ME rather than an MLE pathway along *E. faecalis* evolution might be related to NADH generation via the malic but not the malolactic reaction (Fig. 1A). This extra contribution to reducing power could be redirected to different metabolic routes and, in that way, may confer an adaptive advantage to this bacterium.

Materials and methods

Bacterial strains and growth media

Es. coli DH5α (Bethesda Research Laboratories, CA, USA) was used as a general cloning host while *Es. coli* BL21 (DE3) was used for expression of recombinant CitM and MaeE proteins. *Es. coli* EJ1321, a mutant strain lacking ME and phosphoenolpyruvate carboxykinase activities [22], was used for complementation studies. *Es. coli* cells were grown aerobically at 37 °C in LB medium and transformed as previously described [34]. Complementation tests were performed in MSMYE medium [35] supplemented with 80 mM succinate and 50 μM IPTG. Culture growth was monitored by measuring absorbance at 660 nm in a PowerWave™ XS Microplate reader (BioTek, BioTek Instrument Inc., Vermont, USA). *E. faecalis* JH2-2 cells were routinely grown at 37 °C without shaking in LB basal medium (Difco, New Jersey, USA) or with the addition of 35 mM malate (LBM). The initial pH value was adjusted with an HCl solution. Alternatively, M17 (Difco) or Milk medium [36] were employed when indicated. Kanamycin (50 $\mu g \cdot mL^{-1}$), ampicillin (100 $\mu g \cdot mL^{-1}$) and erythromycin (5 and 100 $\mu g \cdot mL^{-1}$ for *E. faecalis* and *Es. coli*, respectively) were added to the medium when necessary.

Construction of *E. faecalis* JH2-2 MaeE defective strain

The strain was constructed by interrupting the *maeE* gene by a single recombination event using the thermosensitive vector pGh9 [37]. An internal fragment of *maeE* was amplified by PCR using chromosomic DNA of *E. faecalis* JH2-2 as template. The forward primer (5′-CTGCCGCTAAAGC TTCATCAGG-3′) contains a *Hind*III, and the reverse primer (5′-CCGAAGAAAGAATTCAAACGG-3′) introduced an *Eco*RI site. The amplimer was digested with these two enzymes and cloned into the corresponding sites of pGh9 vector. The resulting plasmid, pGh9-L, was used to transform *Es. coli* EC101. From that strain, pGh9-L was isolated and then electroporated into *E. faecalis* JH2-2 strain as described elsewhere [38]. The transformant strain was grown overnight at the permissive temperature of 30 °C in LB plus glucose with erythromycin 5 $\mu g \cdot mL^{-1}$. The saturated culture was diluted 500-fold into fresh medium and incubated at the restrictive temperature of 37 °C at which plasmid replication is disabled. When the culture reached $D_{660} = 0.5$, serial dilutions were plated on LB plus glucose and antibiotic. The interruption of *maeE* was confirmed by PCR.

Cloning, expression and complementation

The open reading frames corresponding to CitM and MaeE from *E. faecalis* JH2-2 were amplified by PCR using a forward primer (5′-GTGACCATATGTTAGAAGAAGTTC TAG-3′ and 5′-GGAAAATCATATGTCAACAAAAGAT G-3′, respectively) containing an *Nde*I restriction site and a reverse primer (5′-TGTCGGATCCTTTTACGTCCCTTC-3′ and 5′-ATTAATCGGATCCACAGTTCTATTTACTC-3′, respectively) containing a *Bam*HI restriction site. The amplified DNA fragment was ligated to the *Nde*I and *Bam*HI sites of a pET28a expression vector (Novagen, Darmstadt, Germany) yielding pET-CitM and pET-MaeE plasmids, respectively.

To obtain pQE-CitM and pQE-MaeE plasmids, recombinant CitM and MaeE encoding genes were amplified by PCR using pET-CitM and pET-MaeE as templates. The forward primer (5′-CACGGATCCAGCAGCGGCCTGGT G-3′) contains a *Bam*HI restriction site and the reverse primer (5′- CACGTCGACTTTTACGTCCCTTC-3′ or 5′-CA CGTCGACTAATTTGTTTCTTTG-3′, respectively) an *Sal*I restriction site. The corresponding amplimers were purified, digested with *Bam*HI and *Sal*I and finally cloned into the same sites of the pQE30 vector (Qiagen, CA, USA), thus yielding pQE-CitM and pQE-MaeE. Consequently, each of these plasmids contains a copy of the heterologous gene with almost the same N-terminal coding region with respect to the proteins expressed from pET28 vectors but in this case under the control of T5 promoter. In order to tightly regulate T5 promoter expression, EJ1321 was first transformed with pREP4 plasmid, which carries the $lacI^q$ gene. Next, EJ1321 (pREP4) strain was successfully transformed with plasmid pQE-CitM, pQE-MaeE or pQE30 (empty vector).

Purification of recombinant proteins

To obtain high levels of soluble recombinant His-tagged CitM or MaeE proteins, *Es. coli* BL21 (DE3) cells carrying

plasmid pET-CitM or pET-MaeE, respectively, were grown in LB at 37 °C until $A_{660} \sim 0.6$. At this point, cells were induced by addition of 0.5 mM IPTG and incubated at 23 °C for 20 h with slow shaking (25 r.p.m.). Cultures (1.5 L) were then harvested by centrifugation and resuspended in ice-cold A1 buffer [175 mM NaAc pH 6.0, 5 mM $MnCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10% glycerol] for CitM or A2 buffer (50 mM Tris/HCl pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 150 mM NaCl and 3 mM PMSF) for MaeE. Cells were disrupted using a French Press and cell debris was removed by centrifugation as previously described [7]. After addition of 150 mM NaCl and 25 mM imidazole to the CitM extract, both proteins were purified from the soluble fraction by affinity chromatography using an Ni–nitrilotriacetic acid column according to the protocol recommended by Novagen. CitM and MaeE eluted at a 100 mM imidazole concentration. The purified enzymes were then dialysed against their respective resuspension buffers (A1 or A2) supplemented with 20% glycerol and finally stored at −80 °C for further studies. Protein concentrations were determined by the Lowry method using bovine serum albumin as standard.

Enzyme activity assays

OAA, $MnCl_2$, NaAc, HAc, NAD^+ and NADH were purchased from Sigma (St Louis, MI, USA). L-Malate and all other chemicals and reagents were obtained from commercial sources and were high purity. Enzymatic assays were performed in a Jasco UV/Vis V-530 spectrophotometer at 30 °C and optimum pH in 500-μL reaction buffer using a 10-mm path length cell, and 6.7 μg CitM or 3.3 μg MaeE aliquots.

OAD activity was determined following OAA decarboxylation under standard conditions (50 mM NaAc–HAc buffer and 20 mM $MnCl_2$) by measuring the decrease of the enolic OAA absorbance at 280 nm [7]. The reported OAD activity was corrected considering the spontaneous decarboxylation of OAA catalysed by the presence of the divalent metal ion. The optimal pH value for OAD activity was determined using 50 mM NaAc–HAc buffer (1 mM OAA and 10 mM $MnCl_2$) ranging between pH 3.7 and 5.6.

Malic activity was determined by measuring the increase in NADH absorbance at 340 under standard conditions (50 mM Tris/HCl buffer, 0.1 mM $MnSO_4$, 1.0 mM NH_4Cl and 0.5 mM NAD^+). The optimal pH value for malic activity was determined under standard conditions with 1.5 mM malate and 50 mM Tris/HCl buffer ranging between pH 7.3 and 9.4.

$K_{m,substrate}$ and k_{cat} for the enzymatic reactions were determined considering theoretical molecular weights. Measurements were carried out with varying substrate concentration while keeping a saturating Mn^{2+} concentration. Experimental data were evaluated by the Michaelis–Menten equation and non-linear regression. The effects of different metals, metabolites and substrate analogues on the enzymatic activities were tested by addition of the appropriate amounts of each compound in the assay mixture as indicated (Tables 1 and 2).

Gel electrophoresis and zymograms

The purity of the enzyme preparations was estimated by using a modified Laemmli gel [39] that was subsequently stained with Coomassie brilliant blue R-250. For native PAGE, gels (7.5%) were electrophoresed at 150 V and 10 °C. Gels were then analysed by Coomassie staining or detecting malic activity by incubation at room temperature in a solution containing 200 mM Tris/HCl pH 8.5, 200 mM L-malate, 20 mM Mn^{2+}, 10 mM NAD^+, 0.1 mg·mL^{-1} nitroblue tetrazolium and 5 μg·mL^{-1} phenazine methosulfate [4].

Malate quantification

Malate concentration in culture supernatants was determined by the appearance of NADH in a reaction catalysed by MaeE. This is based on the fact that NADH levels are proportional to the remaining malate in the supernatant of each culture. Reactions were performed using microplates in a final volume of 200 μL. Enzymatic reactions were started by the addition of supernatant (4 μL) to 196 μL of reaction buffer (50 mM Tris/HCl pH 8.5, 0.1 mM $MnCl_2$, 1.0 mM NH_4Cl, 0.5 mM NAD^+ and 1.3 μg of MaeE). After incubating for 10 min at 30 °C NADH production was determined spectrophotometrically by measuring A_{340} with a PowerWave XS (BioTek) microplate reader. The concentration of malate per well was calculated from the regression equation for a standard curve.

Loading of cells with the CDCFD probe

Cells were first grown in batch culture in LBM medium at pH 7.0. Cultures were then harvested by centrifugation after reaching their exponential growth phase at A_{660} between 0.6 and 0.8 and washed once with 50 mM Hepes buffer pH 8.0. Harvested cells were then loaded with the pH-sensitive fluorescent probe 5-(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFD) (Biotium, CA, USA) as previously described [40]. Briefly, 0.1 mM CDCFD solution was added to the cell suspension and incubated for 10 min at 30 °C, washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0, 5.5 or 4.5) and finally stored in ice until used.

Cytoplasmic pH measurements

For each experiment, CDCFD-loaded cells (approximately 10^9 UFC) were suspended in 2 mL of 50 mM potassium phosphate buffer pH 4.5, 5.5 or 7.0 and introduced in a

3 mL quartz-cuvette (1-cm path length) equilibrated at 30 °C. Samples were mixed by using a magnetic stir bar and the fluorescent signal was monitored every second in a fluorescence spectrometer (Perkin Elmer LS 55). Excitation wavelength was 490 nm and fluorescent emission was recorded at 525 nm (slit widths were 5 nm). Cytoplasmic pH values were determined from the fluorescence signal as previously described [41]. Cytoplasmic and external pH values were equilibrated at the end of each assay by addition of 1 mM valinomycin, 1 mM nigericin and 2% v/v Triton X-100. Calibration curves were determined in 50 mM potassium phosphate buffer with pH values between 3.0 and 11.0. pH was adjusted with either NaOH or HCl.

Bioinformatic analysis of MEF homologs

Protein sequences of MEF homologues were obtained from UniProtKB and RefSeq databases by BLASTP using YtsJ, CitM and MleA from *B. subtilis*, *L. lactis* and *O. oeni*, respectively, as query. Multiple alignments were performed using MEGA software version 4.0 [42]. The phylogeny of MEF proteins was inferred from 75 aligned primary sequences (Table S1), using the neighbour-joining method by means of the same application. The reliability of the inferred tree was tested by the bootstrap technique with 1000 replicates [43].

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Supporting information

The following supplementary material is available:
Table S1. MEF protein members represented in the phylogenetic analysis.

This supplementary material can be found in the online version of this article.

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