International Journal of Food Microbiology 218 (2016) 27–37

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Aroma compounds generation in citrate metabolism of *Enterococcus faecium*: Genetic characterization of type I citrate gene cluster

Gabriela P. Martino [1], Ingrid M. Quintana [1], Martín Espariz, Victor S. Blancato, Christian Magni *

Laboratorio de Fisiología y Genética de Bacterias Lácticas, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 590, S2002LRK Rosario, Argentina

A R T I C L E   I N F O

Article history:
Received 15 June 2015
Received in revised form 24 September 2015
Accepted 11 November 2015
Available online 14 November 2015

Keywords:
Citrate metabolism
Citrate lyase
Oxaloacetate decarboxylase
Diacetyl/acetoin
Enterococcus faecium

A B S T R A C T

Enterococcus is one of the most controversial genera belonging to Lactic Acid Bacteria. Research involving this microorganism reflects its dual behavior as regards its safety. Although it has also been associated to nosocomial infections, natural occurrence of *Enterococcus faecium* in food contributes to the final quality of cheese. This bacterium is capable of fermenting citrate, which is metabolized to pyruvate and finally derives in the production of the aroma compounds diacetyl, acetoin and 2,3 butanediol. Citrate metabolism was studied in *E. faecium* but no data about genes related to these pathways have been described. A bioinformatic approach allowed us to differentiate cit^{-} (no citrate metabolism genes) from cit^{+} strains in *E. faecium*. Furthermore, we could classify them according to genes encoding for the transcriptional regulator, the oxaloacetate decarboxylase and the citrate transporter. Thus we defined type I organization having CitI regulator (DeoR family), CitM cytoplasmic soluble oxaloacetate decarboxylase (Malic Enzyme family) and CitP citrate transporter (2-hydroxy-carboxylate transporter family) and type II organization with CitO regulator (GntR family), OAD membrane oxaloacetate decarboxylase complex (Na^{+}-transport decarboxylase enzyme family) and CitH citrate transporter (CitMHS family). We isolated and identified 17 *E. faecium* strains from regional cheeses. PCR analyses allowed us to classify them as cit^{-} or cit^{+}. Within the latter classification we could differentiate type I but no type II organization. Remarkably, we came upon *E. faecium* GM75 strain which carries the insertion sequence IS256, involved in adaptative and evolution processes of bacteria related to *Staphylococcus* and *Enterococcus* genera.
In this work we describe the differential behavior in citrate transport, metabolism and aroma generation of three strains and we present results that link citrate metabolism and genetic organizations in *E. faecium* for the first time.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Enterococcus faecium is a gram positive bacterium occurring in diverse niches. It is a commensal bacterium of animal and insect gastrointestinal tract, but it can also be found in fermented food such as dairy or meat derived products and as free living cells in soil, plants and water (Devriese et al., 2006; Lebreton et al., 2014). In the last decade *E. faecium* has emerged as an important nosocomial pathogen, frequently acquiring even more antibiotic resistances than *Enterococcus faecalis* (Arias and Murray, 2012). Vancomycin- and ampicillin-resistant strains are the most common pathogens involved in infections due to contaminated devices such as catheters, central lines and ventilators. As regards food industry, one of the main problems of *Enterococcus* participation in different ripening processes is its production of biogenic amines (Giraffa, 2003; Ogier and Serror, 2008; Suarez et al., 2012, 2013).

Certainly, aspects related to pathogenicity and antibiotic resistance as well as biogenic amines production are not desired properties in food microorganisms. Nevertheless, *Enterococcus* strains have industrial interest (Giraffa, 2003; Foulquié Moreno et al., 2006; Ogier and Serror, 2008) and in fact, some selected strains have been proposed as probiotic or as adjunct starter microflora (Foulquié Moreno et al., 2003; Franz et al., 2011). Also, the presence of *E. faecalis* and *E. faecium* in Mediterranean and South American traditional cheeses has been reported along with their positive contribution to flavor formation. This is related to citrate fermentation and the resulting aroma compounds production (Blancato et al., 2008; Cabral et al., 2007; De Vuyst et al., 2011; Rea and Cogan, 2003, Sarantinopoulos et al., 2001, 2003; Vaningelgem et al., 2006).

C4-metabolites (acetoin, diacetyl and 2,3 butanediol) are synthesized from citrate fermenting pathways and are involved in flavor development. Diacetyl and acetoin provide dairy products with their typical buttery flavor. Relevance of citrate metabolism in *E. faecium* was previously studied (Cabral et al., 2007; De Vuyst et al., 2011; Rea and Cogan, 2003, Sarantinopoulos et al., 2001, 2003; Vaningelgem et al., 2006), as well as its main final metabolites pyruvate, acetate,

* Corresponding author at: Laboratory of Genetics of Lactic Acid Bacteria, Suipacha 590, S2002LRK Rosario, Argentina.
E-mail address: magni@ibr-conicet.gov.ar (C. Magni).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.11.004
0168-1605/© 2015 Elsevier B.V. All rights reserved.

CO_2 and the minor amounts of formate, ethanol, acetaldehyde, acetoin, and diacetyl which may be obtained depending on growth conditions (Fig. 1). Although the final products have been well established and studied, genes involved in citrate metabolism of *E. faecium* have not.

Our laboratory has made exhaustive efforts in characterizing the organization, function and regulation of genes involved in citrate fermentation and C4-metabolites production in Lactic Acid Bacteria (LAB). The first step in this metabolic pathway involves a specific citrate transporter that makes citrate available to the citrate lyase complex, which catalyzes in turn the breakup of citrate into acetate and oxaloacetate (García-Quintans et al., 2008, Magni et al., 1994, 1996, 1999; Martin et al., 2004). The next step consists on the decarboxylation of oxaloacetate by the oxaloacetate decarboxylase enzyme, thus obtaining pyruvate and CO_2 (Sender et al., 2004). As a result, two genetic organizations of the *cit* cluster were established in LAB; i) type I, which occurs in *Lactococcus lactis* (Martin et al., 2005), *Leuconostoc mesenteroides* (Bekal-Si et al., 1999) and *Weissella paramesenteroides* and involves the transcriptional activator CitI, a member of the DeoR family (*citI*) (Martin et al., 1999, 2000, 2005), the soluble oxaloacetate decarboxylase CitM (*citM*) (Espariz et al., 2011; Sender et al., 2004) and the citrate transporter CitP (*citP*) (Magni et al., 1994) and ii) type II, present in *E. faecalis* and *Lactobacillus casei* involving the activator CitO, a GntR family member of transcriptional factors (*citO*) (Blancato et al., 2008; Mortera et al., 2013), the membrane pump Na^+ complex oxaloacetate decarboxylase (Repizo et al., 2013) and CitH (Blancato et al., 2006; Mortera et al., 2013) (Fig. 2A).

Regulation of type I *cit* cluster in *L. lactis* involves the induction of *cit* catabolic genes at low pH but no catabolic repression (García-Quintans et al., 2008). In *W. paramesenteroides*, type I cluster is induced in the presence of citrate and influenced neither by the acidification of external medium nor by the presence of glucose (Martin et al., 2000, 2005). Conversely, type II *cit* genes in *E. faecalis* are induced in the presence of citrate and downregulated by PTS sugars via CcpA dependent and independent repression mechanisms (Blancato et al., 2008; Suárez et al., 2011).

Moreover, we found differences in genetic organization and regulatory mechanisms of the aroma anabolic pathway. In *L. lactis* acetoin, diacetyl and 2,3 butanediol production depends on genes *alsS* (coding for an α-acetolactate synthase), *aldB* and *aldC* (two α-acetolactate decarboxylase isoforms) and *butA* and *butB* (that codes for a butanediol dehydrogenase and diacetyl reductase, respectively) (García-Quintans et al., 2008; Zuljan et al., 2014). These genes are induced at low pH together with the chromosomal and plasmidic genes involved in citrate metabolism.

On the other hand, in *E. faecalis* aroma compound synthesis relies on the bicistronic operon *alsSD* (which codes for the α-acetolactate synthase and the decarboxylase, respectively). In this case the operon expression is enhanced by the addition of pyruvate to growth media (Repizo et al., 2011).

The aim of this study was to initiate the genetic characterization of citrate metabolism in *E. faecium*, a microorganism present in natural starter cultures involved in spontaneously fermented food. We present evidence about the diversity of genetic organizations of the *cit* cluster in

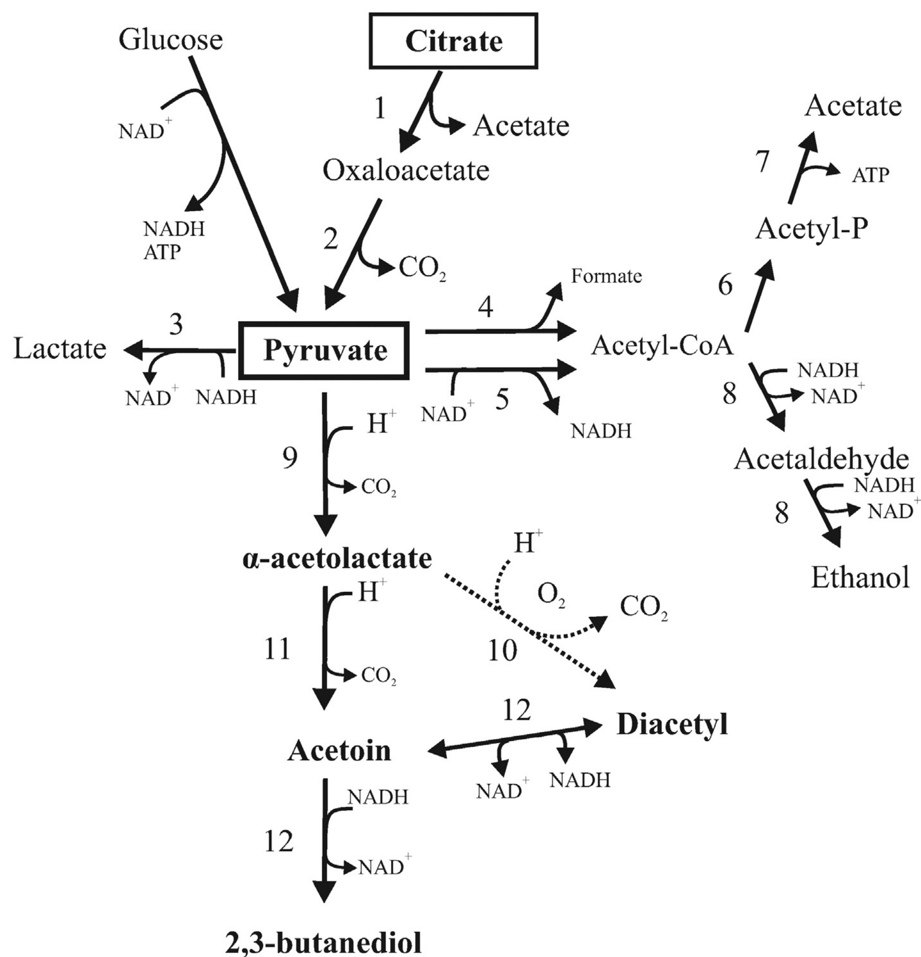

Fig. 1. Representation of the citrate metabolic pathway and aroma compound generation in *Enterococcus faecium*. Enzymes involved in citrate metabolism: 1, citrate lyase; 2, oxaloacetate decarboxylase. Enzymes involved in pyruvate metabolism: 3, lactate dehydrogenase; 4, pyruvate formate lyase; 5, pyruvate dehydrogenase; 6, phosphotransacetylase; 7, acetate kinase; 8, alcohol dehydrogenase; 9, α-acetolactate synthase, 10, non-enzymic oxidative decarboxylation; 11, α-acetolactate decarboxylase and 12, diacetyl and (or) acetoin reductase.

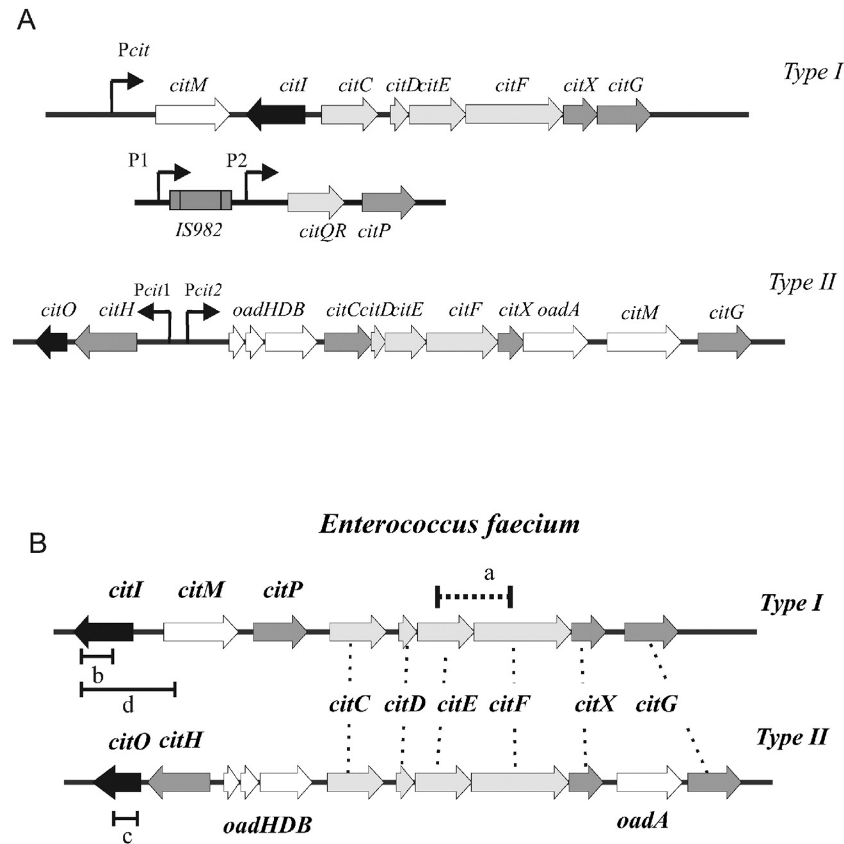

Fig. 2. Genetic architecture of *cit* cluster in Lactic Acid Bacteria. A) Type I organization, present in *Lactococcus lactis*, *Weissella paramesenteroides*, *Leuconostoc mesenteroides* and type II, present in *E. faecalis* and *Lactobacillus casei*. B) Type I and II organizations found in *E. faecium*. a, b, c and d amplicons used in the identification and characterization of the cheese isolated strains are indicated. See more details in the text.

E. faecium strains isolated from artisanal and manufactured cheeses. This paper also shows for the first time that different *E. faecium* strains can have differential contributions to aroma generation during the cheese making process. Thus, our study contributes to the rational selection of strains with possible use as adjunct culture.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Enterococcal strains used in the present study are shown in Table 1. Cultures of *E. faecium* were routinely grown at 37 °C without shaking in 100 ml sealed bottles containing 20 ml of LB medium, supplemented with 30 mM glucose (LBG). Overnight cultures prepared in this way were used to inoculate fresh LB supplemented with different compounds at concentrations indicated in each case. Inoculums were diluted to an initial OD_{600} of approximately 0.2 for cultures adjusted to initial pH 7.0.

2.2. Isolation and biochemical identification of Enterococcus genus

1 g samples of different semi-hard commercial and regional cheeses (Pampeana region) were homogenized in 10 mL of 2% (w/v) sterile sodium citrate solution. Serial dilutions were made and 0.1 mL was plated on solid MRS medium (MRS 0.5% glucose). The plates were grown at 37 °C for 48 h until appearance of isolated colonies.

Gram-positive, catalase-negative cocci were selected for further characterization. *Enterococcus* spp. were initially selected based on the following biochemical test: growth at high osmolarity (NaCl 6.5% w/v), temperature (45 °C), or pH value (pH 9.6) and also growth in bile esculin (40%) agar medium. In addition, tellurite tolerance, leucine aminopeptidase, and pyrrolidonilarilamidase enzyme tests were used for biochemical identification of *Enterococcus* strains (Suarez et al., 2012). Stock cultures were kept in M17 broth plus glycerol (70:30) at −80 °C and were cultured twice in the respective broths for activation.

Genetic variability of isolates was analyzed by Randomly Amplified Polymorphic DNA (RAPD) as described in Suarez et al., 2012. Finally, representative isolates were analyzed by 16S rRNA gene sequencing in order to confirm assignment to the *E. faecium* species. Briefly, 2 μL DNA (to a final concentration of 0.2 ng/μl) was used as a template for the PCR and primers based on conserved regions of the 16S rRNA gene, 16S-1389-rev (5'ACGGGCGGT GTGTACAAG 3´), and 16S-341-fwd (5'CCTACGGGAGGCAGCAG 3´) were used to amplify a 1066 bp region (Suarez et al., 2012).

2.3. Citrate-fermenting capacity

Kempler and McKay medium allows selection of citrate-fermenting (blue) colonies from non citrate-fermenting (white) colonies (Kempler and McKay, 1980). The composition of this medium was modified in the following way: peptone 10 g/L, casein 2.5 g/L, beef extract 5 g/L, yeast extract 2.5 g/L, agar 15 g/L pH was adjusted to 6.5 and sterilized for 15 min at 115 °C. Then 10 ml of potassium ferricyanide (10% w/v) and 10 ml of sodium citrate and ferric citrate (2.5% w/v each) were added to the medium. Plates were incubated during 7 days at 30 °C. *L. lactis* CRL264 and *E. faecalis* JH2-2 were used as positive control (blue colonies) (Magni et al., 1994; Blancato et al., 2008).

2.4. Growth curves

For growth curves experiments, *E. faecium* IQ110, IQ23 and GM75 were cultivated in sterile 96-well microplates (Cellstar) in a total

Table 1
Phenotypic and genotypic analysis of *Enterococcus faecium* strains.

Strain	KM	Aggregation phenotype	CL amplicon a	*citI* amplicon b	*citO* amplicon c	IR amplicon d	Type
IQ22	−	−	+	+	−	1244 pb	I
IQ23	−	+	+	+	−	1244 pb	I
IQ25	−	+	+	+	−	1244 pb	I
IQ101	−	−	−	−	−	−	*cit*$^-$
IQ103	−	−	−	−	−	−	*cit*$^-$
IQ110	−	−	−	−	−	−	*cit*$^-$
IQ121	−	−	−	−	−	−	*cit*$^-$
IQ167	−	−	+	−	−	−	*cit*$^+$
IQ336	−	+	−	−	−	−	*cit*$^-$
GM70	−	−	+	+	−	1244 pb	I
GM71	−	+	+	+	−	1244 pb	I
GM72	−	−	−	−	−	−	*cit*$^-$
GM75	−	−	+	+	−	2576 pb	I
GM152	−	+	−	−	−	−	*cit*$^-$
GM156	−	−	−	−	−	−	*cit*$^-$
GM203	−	−	−	−	−	−	*cit*$^-$
GM241	−	−	−	−	−	−	*cit*$^-$

KM: blue color production on Kempler and McKay medium; CL: *citF-citE* region; IR: intergenic region, amplicon a, b, c and d are indicated in Fig. 2, +: positive PCR reaction, −: negative PCR reaction.

volume of 200 µl at 37 °C as previously described (Repizo et al., 2011). Exponentially growing cultures were diluted to an initial OD_{600} of approximately 0.2 in LB broth containing the indicated substrates (glucose, citrate or none) at initial pH value of 7.0. Glucose was added to a final concentration of 30 mM and citrate of 15 mM. Once cultures had been set up, OD_{600} was automatically registered every 15 min in a PowerWave XS (BioTek) microplate reader. Data presented are the mean values of three independent growth curves.

2.5. Aroma compounds detection

Diacetyl and acetoin production was first determined by Voges Proskauer (VP) qualitative test. Exponentially growing cultures were diluted (initial OD_{600} of 0.1) in LB broth or LB supplemented with glucose 30 mM, pyruvate 100 mM or citrate 15 mM, at initial pH value of 5.5, to allow pyruvate internalization. After 5 h of incubation at 37 °C, the presence of diacetyl and acetoin in the supernatant (SN) of cultures was determined as follows: 1 ml of SN was mixed with 0.6 ml of α-naftol 5% w/v of ethanol 96% and 0.2 ml of potassium hydroxide 40% w/v. The acetoin present in each culture is oxidized in the tested conditions to form diacetyl. All diacetyl present in the culture reacts with peptone from LB to produce color. Positive reaction was recognized by red color.

Amounts of diacetyl and acetoin produced in the tested conditions were measured by an adaptation of Fertally and Facklam method, in the VP quantitative test (Fertally and Facklam, 1987). Briefly, strains were grown in LB or LB with different supplements, glucose 30 mM (LBG), citrate 15 mM (LBC), pyruvate 100 mM (LBP), citrate and glucose (LBCG), pyruvate and glucose (LBPG), ribose 30 mM (LBR) and ribose and citrate (LBRC), for 5 h at 37 °C. After this incubation, resting cells of each strain and condition assayed were prepared as follows. Cells were harvested by centrifugation for 12 min at 5500 rpm at 4 °C. Pellets were washed twice with 50 mM potassium phosphate (KPi) buffer pH 5.8, and finally resuspended in the same buffer pH 7.0 or 5.8 in a total volume of 1.5 ml and at a final OD_{600} of 1.5. Resting cells obtained by this way were incubated with citrate (15 mM), pyruvate (100 mM), glucose (30 mM) or ribose (30 mM) at 37 °C without agitation for 60 min. The supernatant of each culture (175 µl) was transferred to 96-well microplates and 25 µl of a freshly prepared solution containing 15% (w/v) α-naphthol and 0.5% (w/v) creatine in 7.5 M NaOH was added. After 10 min of incubation, the microtitre plate was loaded into the PowerWave XS (BioTek) microplate reader and A_{540} was recorded. The concentration of aroma compounds per well was calculated from the regression equation for a diacetyl standard curve. Results are presented as the mean and SD of assays performed in triplicate.

2.6. Genotypic identification of the cit cluster present in E. faecium

Detection of citrate fermenting strains was achieved by identification of the citrate lyase complex. We used degenerated oligonucleotides CL_Up (5′ AYKCRATYATGTTTGAYYTADAAGATGC 3′) and CL_Down (5′ AAATGRTGRTGRAAWGARATSGTCATRCC 3′) that amplified a sequence of 1000 bp corresponding to a 5′ and a 3′ fragment of genes encoding for the α chain of CitF and the β subunit CitE, respectively. The protocol used for the PCR reaction was a denaturation temperature of 95 °C for 30 s, then an annealing temperature of 53 °C for 30 s and the elongation step was performed at 72 °C for 2 min.

To discriminate type I from type II citrate fermenting strains, two pairs of specific oligonucleotides were used; citI_Up (5′ TGATGAATTCACCATTGATATTGAAGG 3′) and citI_Lo (5′ AATTAAGCTTGCCAATGTGGTTGC 3′) amplified a 1440 bp fragment from the transcriptional regulator *citI*, and the pair of primers citO_UP (5′ GGTACGCGAATTCACGAAAAAG 3′) and citO_Lo (5′ GAGGAAGCTTTGGCAATATCACG 3′) was used for amplification of a fragment of 1331 bp from the transcriptional regulator *citO*. PCR reaction was performed at a denaturation temperature of 95 °C for 30 s, then an annealing temperature of 55 °C for 30 s and an elongation step at 72 °C for 2 min. Finally, the intergenic region of *citI* was analyzed using oligos citIf (5'ACAGGGATCCTTTGGAGGATTTTACATGAC 3´) and citMr (5'ACAGGGATCCGGCTTCTGTTGTTTCC 3´), which amplify the region comprised between *citI* and *citM*, including *citI* sequence. PCR protocol was performed initially with 2 min of denaturation at 95 °C and 30 cycles with a denaturation step at 95 °C for 1 min, annealing at 59 °C for 45 s and elongation at 72 °C for 1 min 20 s. All PCR reactions were performed using 2.5 µl of total genomic DNA to a final concentration of 0.2 ng/µl in a final volume of 25 µl.

2.7. Citrate transport assay

Bacterial cultures were grown in LBG or LBC to an OD_{600} of 0.6, harvest by centrifugation, and washed in 50 mM potassium phosphate buffer, pH 5.8. Cultures were resuspended to 10 OD/ml and stored in ice until use. Transport was assayed over a 10-min period with 688 pM [1, 5 − ^{14}C] citrate (109 mCi/mmol) at 30 °C. For this, a dilution of the resuspended cells was made to a final OD of 3 and incorporation of [^{14}C] citrate was measured as previously described (Magni et al., 1996; Blancato et al., 2006).

2.8. Internal pH measurements using the fluorescent probe BCECF in E. faecium

Loading of the cells with the fluorophore was performed as previously explained (Esparíz et al., 2011), with the following modifications; cells were washed twice with 50 mM potassium phosphate buffer (KPi) pH 5.8 and loaded with the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF) as previously described (Magni et al., 1999). The suspension was incubated for 5 min at room temperature, then washed three times, resuspended in 50 mM KPi pH 5.8 and finally stored in ice until used. Internal pH changes were followed in the same buffer using the protocol reported by Esparíz et al., 2011. Excitation wavelength was 503 nm and fluorescent emission was recorded at 525 nm (slit widths were 4 and 16 nm, respectively). Cytoplasmic pH was determined from the fluorescence signal as previously described (Magni et al., 1999).

3. Results

3.1. Diversity of the citrate fermenting strains of Enterococcus faecium isolated from cheese

To isolate *E. faecium* strains from cheese, 20 different commercial and artisanal cheeses were processed as described in materials and methods. 75 putative strains were initially isolated as belonging to *Enterococcus* spp., identified as gram-positive cocci, catalase-negative, able to grow at 6.5% w/v NaCl, 45 °C, pH 9.6, in bile-esculin (40%) agar medium, tolerant to tellurite, positive for leucine aminopeptidase, and pyrrolidonilarilamidase enzyme. These strains were analyzed by RAPD-PCR assay and representative strains were selected for 16S rRNA gene sequencing. In a subsequent step, individuals belonging to *E. faecium* species were confirmed by nucleotide sequence analysis using BLAST search tool (Altschul et al., 1990). As a result, 17 different *E. faecium* strains were found (Table 1).

Citrate fermenting strains of Lactic Acid Bacteria are usually differentiated using Kempler and McKay medium (Kempler and McKay, 1980). We expected positive results for the case of citrate fermenting *E. faecium* strain, because we previously use this medium to identify cit^+ strains belonging to *L. lactis*, *W. paramesenteroides*, *L. mesenteroides* and *E. faecalis* (Suárez et al., 2013; Martin et al., 1999 and Blancato et al., 2008). Nevertheless, this experiment was unable to discriminate *E. faecium* species as none of our strains produced the typical blue color of the citrate fermenting phenotype. Since citrate metabolism is linked to the presence of the citrate lyase complex, conserved genes encoding for citrate lyase subunits *citE* and *citF* were looked for by PCR using degenerated oligonucleotides (Materials and methods, Fig. 2B, amplicon a). As shown in Table 1, 7 out of the 17 studied strains were classified as cit^+, as a result of nucleotide sequence analysis of each amplicon obtained.

Moreover, using the available genomic data and gene context analysis (Blancato et al., 2008; Sender et al., 2004) we noticed that it is possible to identify both types of citrate genetic organizations in *E. faecium*. Type I is present in representative *E. faecium* strains 1.230.933 (NZ_ACAS00000000.1), Com15 (NZ_ACBD00000000.1), 1.231.501 (NZ_ACAY00000000.1) and 1,231,502 (NZ_ACAX00000000.1) whereas type II was found in the representative genomes of *E. faecium* strains TX1330 (NZ_ACHL00000000.1), Com12 (NZ_ACBC00000000.1), 1,231,408 (NZ_ACBB00000000.1) and 1,141,733 (NZ_ACAZ00000000.1) (Fig. 2). Therefore, we attempted to discriminate the isolated cit^+ strains by designing 2 different sets of oligonucleotides specific for *E. faecium citI* (amplicon b) or *citO* (amplicon c) genes (representative genes for each organization) (Section 2 and Fig. 2B).

Among our cheese isolated strains, we were able to identify type I genetic organization only (Table 1) with no positive results for type II designed oligonucleotides. IQ167 strain, positive for *citEF* PCR, threw a negative result for both oligonucleotides sets requiring further analysis to be classified. It is important to note that we observed phenotypic differences within *E. faecium* strains. The first of this differences we found it when bacteria where grown in liquid media since a number of isolates formed cellular aggregates which remained insoluble despite mechanical disruption (vortex or pipetting) (Table 1 and Fig. 3B).

Continuing with citrate metabolism characterization of *E. faecium*, 3 representative strains were selected: IQ110 (cit^- no aggregation phenotype) (Fig. 3A), IQ23 (cit^+, aggregation phenotype) (Fig. 3B) and GM75 (cit^+, no aggregation phenotype) (Fig. 3C). First, we analyzed their ability to grow in LB and the effect of the presence of citrate (15 mM) or glucose (30 mM) upon the growth curve pattern and the aroma compounds production once stationary phase was reached (at 5 h) (Fig. 3). All of the strains (IQ110, IQ23 and GM75) were able to grow in LB media alone, showing similar growth curves patterns (Y_{max} of 0.8 ± 0.1, 0.67 ± 0.03 and 0.704 ± 0.007 for IQ110, IQ23 and GM75 respectively), and in this condition aroma compound production was not detected by the used methodology in stationary growth phase. Addition of glucose to LB threw a similar growth pattern for the three studied strains (Fig. 3). Remarkably, as found in previous reports, in this condition *E. faecium* IQ110, IQ23 and GM75 can produce aroma compounds at the stationary phase using glucose as a substrate. Addition of citrate to LB medium (LBC) allowed cit^+ strains (IQ23 and GM75) to significantly increase the Ymax (OD_{600} 1.12 ± 0.04 and 1.30 ± 0.03 in the case of *E. faecium* IQ23 and GM75, respectively), being possible to detect C4 production in the stationary phase (Fig. 3 and Table 2). After addition of citrate to LB medium (LBC), cit^- strain IQ110 had a similar behavior than strains growing in LB alone (OD_{600} 0.69 ± 0.03 in LBC) (Fig. 3 and Table 2) and again no diacetyl or acetoin were produced in this condition.

All these results clearly show that it is possible to isolate *E. faecium* strains from cheese with different contributions to the aroma production. This is of special importance during the cheese ripening process when *E. faecium* is actively involved in the final flavor of the fermented product.

3.2. Citrate transport and metabolism in E. faecium strains isolated from cheese

To analyze citrate transport, representative strains IQ110, IQ23 and GM75 were tested for radioactive citrate uptake in whole cells of *E. faecium* (see materials and methods). As shown in Fig. 4A, *E. faecium* IQ110 cells grown in LBG or LBC did not transport citrate. *E. faecium* IQ23 was able to take citrate in only when cells were grown in the presence of citrate (rate: 13.7 pmol/min) (Fig. 4B). On the contrary, GM75 strain showed significative radioactive citrate uptake in cells grown in both conditions LBG (rate: 2.1 pmol/min) and LBC (rate: 1.7 pmol/min) (Fig. 4C).

Resting cells of *E. faecium* were loaded with pH-sensitive fluorescent probe BCEFC and internal pH was monitored in real time (Magni et al., 1999; Esparíz et al., 2011). As shown in Fig. 4D, and consistent with our previous results of radioactive citrate uptake, resting cells of *E. faecium* IQ110 were unable to alkalinize internal medium after addition of citrate regardless of supplementation of growth media with glucose or citrate (Fig. 4D). Resting cells of *E. faecium* IQ23 and GM75 strains grown in LBG, did not produce internal alkalinization in the fluorometric assay when citrate was added (Fig. 4E). However when both *E. faecium* strains were grown in LBC, resting cells were able to increase internal pH in response to the addition of citrate in the external buffers (Fig. 4E and 4F). Thus, observed internal alkalinization after addition of citrate strongly suggests that the complete pathway is active in both strains. These results show differences related to citrate transport in strains isolated from cheese and strongly suggest that citrate metabolism is induced by the presence of the substrate (citrate) in both strains (IQ23 and GM75).

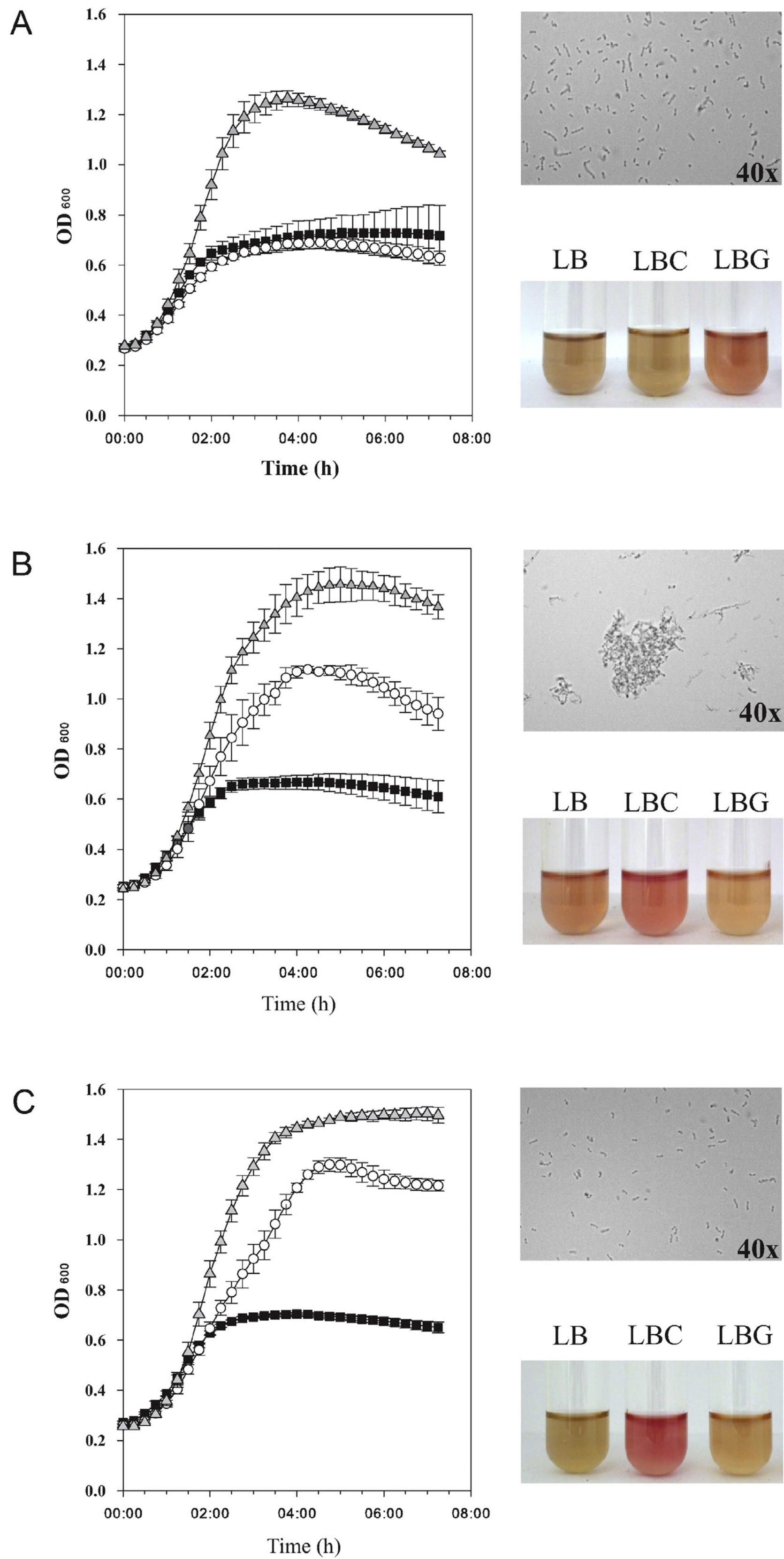

Fig. 3. Growth curves, cells morphology and aroma production for *E. faceium* IQ110 (A), IQ23 (B) and GM75 (C). Strains were grown in LB (black square), LB supplemented with glucose (grey triangle), or LB supplemented with citrate (empty circle). Cell morphology shown in the figure corresponds to strains grown in LB supplemented with citrate at the end of the exponential curve. Aroma production was determined by Voges Proskauer reaction in the stationary phase. Cells were grown in LB medium with supplemented with 15 mM citrate or 30 mM glucose.

Table 2
E. faecium IQ110, IQ23 and GM75 growth in LB, LBG and LBC.

Strain	Medium	Supplement	μ_{max} (h^{-1})	OD_{max}	VP
IQ110	LB	–	0.27 ± 0.03	0.8 ± 0.1	–
	LB	G	0.33 ± 0.01	1.26 ± 0.03	+
	LB	C	0.23 ± 0.01	0.69 ± 0.03	–
IQ23	LB	–	0.30 ± 0.01	0.67 ± 0.03	–
	LB	G	0.40 ± 0.04	1.46 ± 0.07	+
	LB	C	0.30 ± 0.03	1.12 ± 0.04	+
GM75	LB	–	0.26 ± 0.01	0.704 ± 0.007	–
	LB	G	0.404 ± 0.003	1.50 ± 0.03	+
	LB	C	0.282 ± 0.003	1.30 ± 0.03	+

C: citrate 15 mM, G: glucose 30 mM, VP: color production in Voges Proskauer test, – no color, + red color.

3.3. Aroma compound production in *E. faecium* strains

To determine the contribution of citrate metabolism to the aroma production in *E. faecium* we performed resting cells experiments. As it is described in materials and methods, the three strains under study were grown in LB supplemented with different carbon sources. Then, enterococcal cells were harvested and resuspended in buffer and incubated during 1 h after addition of citrate, glucose, pyruvate or ribose. The amount of diacetyl and acetoin produced was determined in the supernatant.

When enterococcal cells were grown in LB medium and then incubated with citrate we detected significant amounts of acetoin and diacetyl only in *E. faecium* GM75 (58.3 ± 1.3 μM) (Fig. 5 and Table 3), whereas no significant amounts of aroma compounds were detected in IQ23 and IQ110 strains (<5 μM). The same negative result was obtained when strains were grown in LBG and resting cells were incubated in the presence of citrate since we were not able to detect C4 production in any case (<5 μM) (Table 3). As shown in Fig. 5 and Table 3, *E. faecium* cells grown in media supplemented with citrate and incubated also in the presence of citrate both *cit*$^+$ strains produced the maximum level of acetoin and diacetyl (165.0 ± 5.2 and 188.1 ± 1.5 μM for IQ23 and GM75, respectively), while we could not detect these compounds in IQ110 strain, as expected.

To further analyze the effect of glucose on aroma production from citrate metabolism we performed resting cells experiments growing *E. faecium* in LBGC and then incubating them with citrate. As shown in Fig. 5A and Table 3 we found more than 8 fold repression in the aroma production in the case of IQ23 strain (165.0 μM/19.1 μM), while GM75 strain showed a partial repression of 2 fold (188 μM/89 μM).

This repressive effect exerted by glucose was compared with the effect of a non-PTS sugar (ribose) when performing the same experiment. Then, we grew the strains in LB supplemented with ribose and citrate (LBCR) and resuspended the cells in the presence of citrate. In this case no repressive effect was exerted by the sugar (Table 3). In this condition enterococcal strains have similar behavior to that shown when grown in the presence of citrate alone.

To investigate the catabolic response to glucose on citrate metabolism we performed the fluorescence assay as previously described. As shown in Fig. 5 we observed again a strong repression given by glucose in IQ23 strain, while no signal was registered after addition of citrate. However when the same experiment was performed for GM75 strain grown in LBGC, the curve showed a slight slope (Fig. 5D) revealing a different repressive effect of glucose in this strain.

Finally, aroma production in *E. faecium* resting cells experiments were performed in order to determine if the observed repressive effect

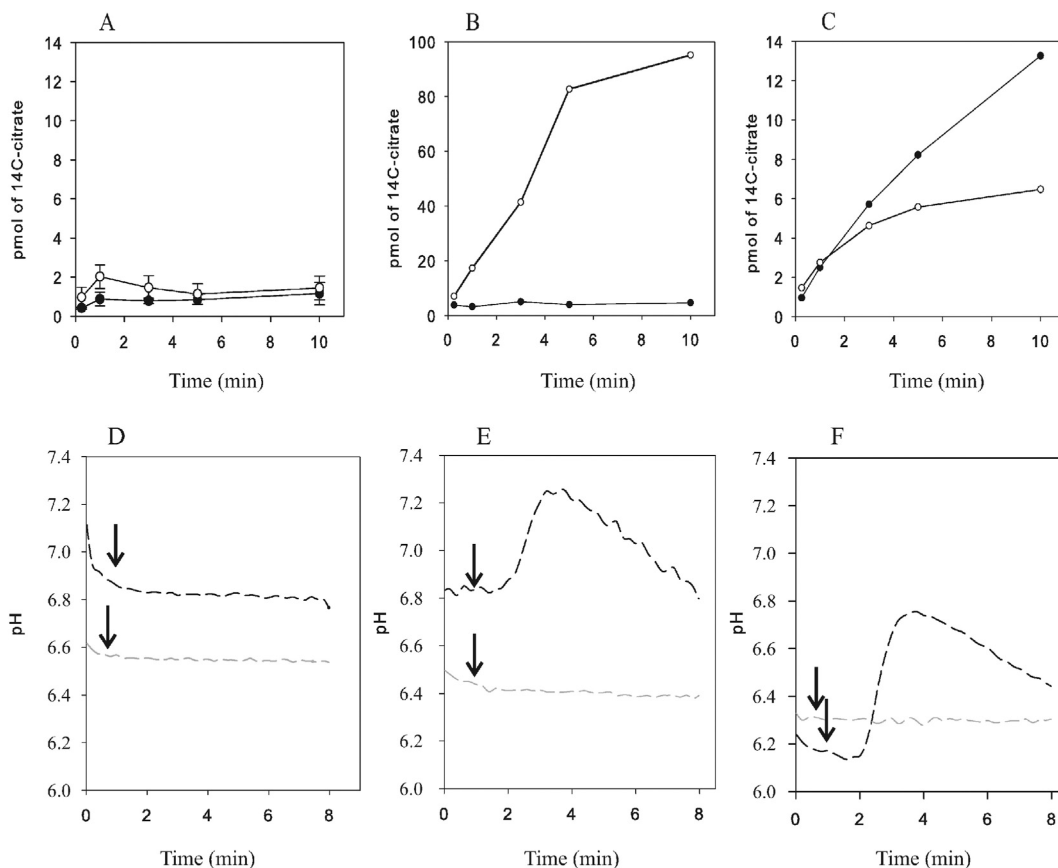

Fig. 4. Citrate transport and metabolism in *E. faecium* strains. (A and D) IQ110 strain, (B and E) IQ23 strain and (C and F) GM75 strain. Top panel: Transport experiments using radioactive citrate. Black circles indicate strains grown in LBG, whereas empty circles indicate strains grown in LBC in all cases. Low panel: Internal pH determination by using fluorescent probe. Black dashed lines represent strains grown in LBC whereas grey dashed lines represent strains grown in LBG in all cases. Citrate addition is indicated by the arrows.

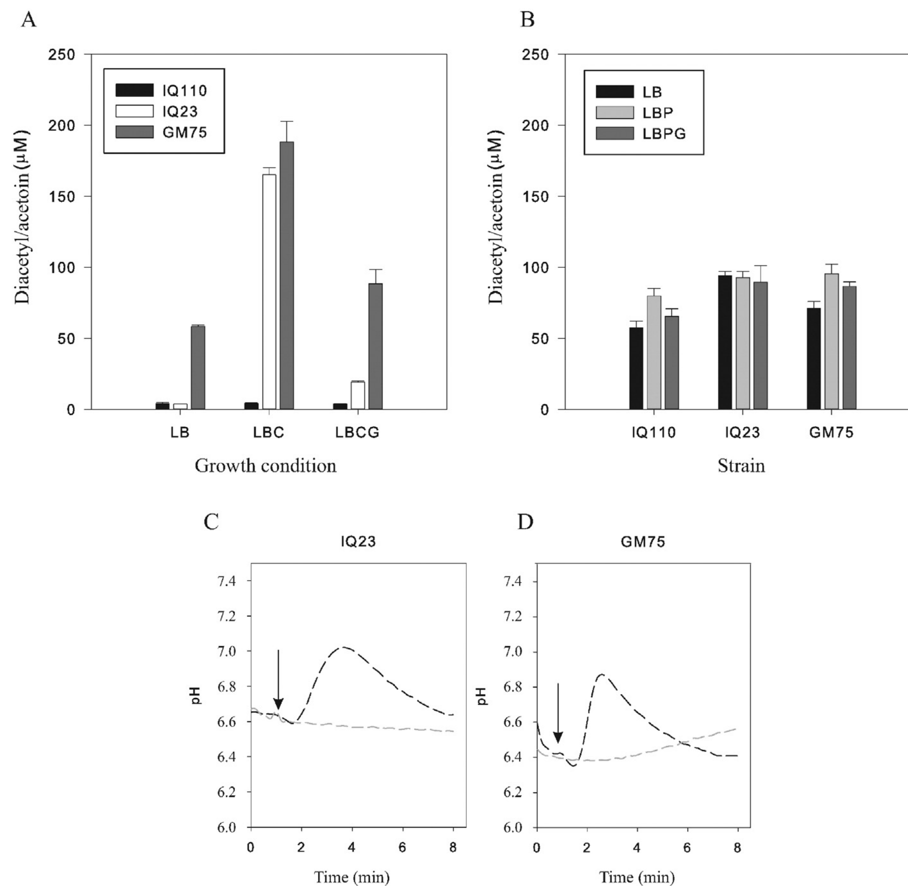

Fig. 5. Catabolic repression on citrate metabolism in selected *E. faecium* strains. A) Cells were grown in LB supplemented with citrate or citrate and glucose, harvested and acetoin and diacetyl production was determined in the presence of citrate. *E. faecium* IQ110 (black bar), IQ23 (white bar) and GM75 (grey bar). B) cells were grown in LB (black bar) or LB supplemented with pyruvate (light grey bar) or pyruvate and glucose (dark grey bar), harvested and acetoin and diacetyl production was determined in resting cells in the presence of pyruvate. In all cases mean values and SD are represented. C) and D) Intracellular alkalinization of *E. faecium* cells, using BCECF probe. Strains were grown in LB supplemented with glucose (30 mM) and citrate (2 mM). Black dashed lines show response to glucose addition whereas grey dashed lines show response to citrate addition to resuspention medium. Citrate addition is shown with an arrow in both cases.

of glucose occurs also in pyruvate metabolism. *E faecium* IQ110, IQ23 and GM75 were grown in LB, LBP and LBPG and incubated in the presence of pyruvate. As shown in Fig. 5B and Table 3, no difference was observed for diacetyl and acetoin production when all *E. faecium* strains were grown in the presence of pyruvate or pyruvate and glucose. These experiments suggest that repression previously observed can be associated with the citrate catabolic route, but not with the diacetyl/acetoin pathway.

Table 3
E. faecium IQ110, IQ23 and GM75 aroma production in resting cells experiments.

Source of cells	Resting cells addition	Diacetyl/acetoin production (μM)		
		IQ110	IQ23	GM75
-	Citrate	n.d.	n.d.	58 ± 1
Glucose	Citrate	n.d.	n.d.	n.d.
Citrate	Citrate	n.d.	165 ± 5	188 ± 2
Citrate + Glucose	Citrate	n.d.	19 ± 1	89 ± 5
Citrate + Ribose	Citrate	n.d.	114 ± 2	211 ± 5
-	Pyruvate	80 ± 5	94 ± 3	71 ± 5
Pyruvate	Pyruvate	80 ± 5	93 ± 4	95 ± 7
Pyruvate + Glucose	Pyruvate	65 ± 5	90 ± 7	87 ± 3
Glucose	Glucose	5.3 ± 0.2	29 ± 3	23 ± 6
Ribose	Ribose	6.2 ± 0.3	n.d.	17 ± 1
Ribose	Citrate	n.d.	n.d.	37 ± 4

n.d.: not detected significant amount of diacetyl/acetoin production (μM) ≤5.0 ± 0.2.

3.4. A full copy of the insertion sequence IS256 is present in the intergenic region of the E. faecium GM75 strain

In order to get more information about the differences observed between *E. faecium* IQ23 and GM75 cit^{+} strains, the intergenic *citM-citI* regions of their *cit* clusters were studied. As shown in Fig. 6, PCR reaction performed using oligonucleotides citIf and citMr (hybridizing in *citI* 3′- and *citM* 5′-end, respectively), gave different sized amplicons for each strain. Sequencing of PCR products revealed amplicons of 1.3 kbp and 2.6 kbp (Fig. 6A), the larger corresponding to *E. faecium* GM75. Detailed in silico analysis allowed us to identify a full length copy of the insertion sequence IS256 (Fig. 6B and 6C) in GM75 intergenic sequence. This IS256 is a widespread mobile element present in multiresistant enterococci and staphylococci. This element is also flanked by imperfect direct repeats as consequence of the transposition mechanism and codes for a putative transposase with a conserved DDE motif (Hennig and Ziebuhr, 2010) (Fig. 6). The *citM-citI* intergenic region of IQ23 is 187 bp long whereas in GM75 strain extends up to 1519 bp. Exact position of the insertion is shown in Fig. 6C.

Both intergenic regions were screened in silico for the presence of putative promoters and operators. One σ^{70} putative promoter was predicted for *citM* (Softberry Bprom, Solovyev and Salamov, 2011) in this region with the −10 (TGTTAaAgT) and −35 (TTtttt) boxes (lowercase indicates deviations from the consensus −10 TGNTATAAT and −35 TTGACA) located 37 and 60 nt upstream *citM* translational start (respectively) which could be directing the expression of the *citM-citG* cluster.

Fig. 6. The IS256 is present in the intergenic *citM-citI* region in the GM75 strain. A) PCR analysis using oligonucleotides citIf and citMr gives two different amplicons, 1.3 kb and 2.6 kb long for IQ23 and GM75 respectively. B) IS256 insertion model in the intergenic region of a wild type strain, resulting in the sequence rearrangement observed in the GM75 strain. The regions amplified with citIf and citMr primers are indicated. C) Integration site of the IS256. Segments of *citM* and *citI* genes are shown. The transposase gene (Tpase) present in the IS256 is flanked by two imperfect inverted repeats (IR) and site directed repeats (dr). *cis* acting sequences are also shown: RBS for *citI* and *citM*, putative operator sequences O1 and O2 for CitI, *cre* site inside of *citI* gene, and the predicted −10 and −35 boxes (see details in the text).

This promoter was found in both *cit*$^{+}$ strains (GM75 and IQ23), so the IS256 may not affect the expression of this group of genes in *E. faecium* GM75 (Fig. 6C).

Aiming to find putative promoters for *citI*, upstream regions of the gene were analyzed in each *cit*$^{+}$ strain. Software predictions for IQ23 threw a σ^{70} site with a −10 extended box (TGGTATAAT) and a −35 (aaGACt) box, at 39 and 62 nt upstream *citI* translational start. Same boxes were found in GM75 at 1372 nt from *citI* ATG (Fig. 6C). This displacement caused by the IS256 could originate a rearrangement of regulatory sites. In fact new promoters closer to *citI* gene were predicted (with lower score) inside non coding sequences of IS256 in *E. faecium* GM75.

In addition, using the *citI* operator consensus sequences described by Martin et al. (O1: TTT TAA A(A/T)A T(A/T)(A/T) (A/G)AA (A/G/T) O2: TTT (A/T)AA A(A/T)A TTT AAA A (Martin et al., 2005) we looked for analogous sites in *E. faecium* IQ23 and GM75. Identical sequences were located in both strains, O1 102 bp (with respect to symmetry center) and O2 81 bp upstream *citM* ATG, only one bp different from the consensus (Fig. 6C).

Since no consensus sequence is defined for *E. faecium cre* site, we searched this region in the IQ23 and GM75 strain using the putative *cis* acting site of related microorganisms such us *Bacillus subtilis* [TGN AA(A/G) CGN (T/A)(T/A)(T/A) CA] (Kim and Chambliss, 1997) and *E. faecalis* [TG(T/A) NAN CGN TN(T/A) CA] and [(T/A)TG (T/A)AA (A/G)CG (C/T)(T/A)(T/A) (T/A)C(T/A)] (Leboeuf et al., 2000; Opsata et al., 2010). We were able to find one putative *cre* site present in both *E. faecium cit*$^{+}$ strains which was located inside of *citI* coding sequence (Fig. 6C).

4. Discussion

Enterococci are part of artisanal and commercial food microbiota; its presence is associated to flavor production, bacteriocin biosynthesis and probiotic properties. Although the presence of *E. faecium* in food is controversial as regards food quality and safety, the capacity of this microorganism to metabolize citrate is one of the most important industrial features related to C4-metabolites production (acetoin, diacetyl and 2,3 butanediol). Nonetheless, in *E. faecium* genetics and regulation of genes and operons involved in these pathways were little explored. In this study, we were able to isolate *E. faecium* strains from artisanal and commercially manufactured cheeses. From those selected we could identify citrate fermenting as well as non-fermenting strains. Our genetic analysis also allowed us to classify *E. faecium* strains belonging to citrate genotype type I, which is related to *cit* operons present in *L. lactis* (Magni et al., 1994; Martin et al., 2004), *W. paramesenteroides* (Martin et al., 1999, 2000), and other LABs (Bekal et al., 1999, Martin et al., 2005). This cluster contains a putative citrate transporter (CitP) belonging to the 2-HCT family (Sobczak and Lolkema, 2005), the soluble oxaloacetate decarboxlyse member of the malic enzyme (Sender et al.,

2004; Espariz et al., 2011) and the activator CitI member of the DeoR transciptional factor (Martin et al., 2005).

On the other hand, despite the screening performed, type II genetic organization was not found among our cheese isolated *E. faecium* strains although there is evidence of this genotype in *E. faecium* as shown by the citrate lyase comparative gene context analysis used in this study to identify citrate fermenting strains.

Our aroma production results clearly show the relevance of citrate metabolism in diacetyl/acetoin biosynthesis. Citrate fermenting capacity (and the presence of *cit* cluster) is required for higher production levels of these industrially important aroma compounds. Citrate lyase negative strain was unable to metabolize citrate and the consequent absence of C4 production from citrate is clearly demonstrated.

Taking into account the genetic scenario found in the citrate fermenting *E. faecium* IQ23, CitI could be involved in intracellular sensing of citrate and in the stimulation of genes involved in its uptake and metabolism. This strain has two transcriptional signals previously described in other species (operator sequences O1 and O2), which could be essential for the DNA-CitI *trans* interaction, and mediation of *cit* genes induction. Also, in this strain we found a PTS-sugar repressive effect on citrate transport and metabolism. In Firmicutes, the growth on efficiently used carbon sources (PTS-sugars) triggers the phosphorylation of HPr at serine-46 (by means of an ATP-dependent HPr kinase). P-Ser-HPr can interact with the global transcription factor CcpA (Deutscher et al., 2006) allowing the complex to bind its DNA *cis* acting sites (catabolite responsive elements, *cre*) repressing the expression of target genes (Schumacher et al., 2004). In growth conditions that lowers the concentration of ATP and glycolytic intermediates the HPr kinase can function as a P-Ser-HPr dephosphorylating, pyrophosphate-forming phosphorylase reducing the amount of functional CcpA-P-Ser-HPr complex and consequently alleviating the repression (Deutscher et al., 2006). With this mechanism bacteria can fine tune the use of less preferred carbon sources.

As shown in fig. 6, the presence of the *cre* site internally located in the coding sequence of the *citI* gene, could indicate that a carbon catabolite repression mechanism may be taking place in IQ23 strain. It is important to point out that *E. faecium* IQ23 strain citrate metabolism is clearly induced by its own metabolite (citrate) and that this pathway might also be repressed by carbon catabolite response. This is the first report where the *cit* cluster type I is under catabolic repression mediated by the master global regulator CcpA.

Moreover, among the *E. faecium* cit^+ type I strains under study, we found one strain (GM75) with a genetic modification due to the insertion of the IS256 element. This insertion sequence was found in *citM-citI* intergenic region and established a fundamental difference between both cit^+ strains. It also raises the question about how this insertion sequence is affecting/interfering with *cit* genes expression since it is located in a region where promoters and regulatory sequences are frequently found. It could be reasonable to attribute the inequalities observed between IQ23 and GM75 strains to the modification of the *cit* promoters location and the addition of new possible promoter sequences.

This is the first report about genetic variability concerning citrate transport and metabolism in *E. faecium* and its relationship with the diacetyl/acetoin pathway involved in aroma generation. Our results show that these bacteria isolated from cheese are subject to considerable diversity in genotypic as well as phenotypic characteristics. In conclusion, the presence of *Enterococcus* (such as *E. faecalis* or *E. faecium*) in artisanal and commercial cheeses can have a positive effect regarding aromatic compound production but also, uncontrolled microorganisms are a potential health risk due to the spread of antibiotic resistances and biogenic amines production. Taking this into account, some selected *E. faecium* citrate fermenting strains could be used as adjuncts in cheese making processes to inhibit undesirable *Enterococcus* strains. This strategy is under investigation in our laboratory.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2014-1513 and PICT 2014-3482, Argentina) and CONICET (PIP 2012-2014). GM and IQ are fellows of CONICET; VSB, ME and CM are researchers of the same institution.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Arias, C.A., Murray, B.E., 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat. Rev. Microbiol. 10, 266–278.
Bekal-Si, A.S., Diviès, C., Prévost, H., 1999. Genetic organization of the *citCDEF* locus and identification of *mae* and *clyR* genes from *Leuconostoc mesenteroides*. J. Bacteriol. 181, 4411–4416.
Blancato, V.S., Magni, C., Lolkema, J.S., 2006. Functional characterization and Me^{2+} ion specificity of a Ca^{2+}-citrate transporter from *Enterococcus faecalis*. FEBS J. 273, 5121–5130.
Blancato, V.S., Repizo, G.D., Suárez, C.A., Magni, C., 2008. Transcriptional regulation of the citrate gene cluster of *Enterococcus faecalis* involves the GntR family transcriptional activator CitO. J. Bacteriol. 190, 7419–7430.
Cabral, M.E., Abeijón Mukdsi, M.C., Medina de Figueroa, R.B., González, S.N., 2007. Citrate metabolism by *Enterococcus faecium* and *Enterococcus durans* isolated from goat's and ewe's milk: influence of glucose and lactose. Can. J. Microbiol. 53, 607–615.
Deutscher, J., Francke, C., Postma, P.W., 2006. How phosphotransferase system related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. 70, 939–1031.
De Vuyst, L., Vaningelgem, F., Ghijsels, V., Tsakalidou, E., Leroy, F., 2011. New insights into the citrate metabolism of *Enterococcus faecium* FAIR-E 198 and its possible impact on the production of fermented dairy products. Int. Dairy J. 21, 580–585.
Devriese, L.A., Baele, M., Butaye, P., 2006. The genus *Enterococcus*: taxonomy. Prokaryotes 4, 163–174.
Espariz, M., Repizo, G., Blancato, V.S., Mortera, P., Alarcon, S., Magni, C., 2011. Identification of malic and soluble oxaloacetate decarboxylase enzymes in *Enterococcus faecalis*. FEBS J. 278, 2140–2151.
Fertally, S.S., Facklam, R., 1987. Comparison of physiologic tests used to identify non-beta-hemolytic aerococci, enterococci, and streptococci. J. Clin. Microbiol. 25, 1845–1850.
Foulquié Moreno, M.R., Rea, M.C., Cogan, T.M., De Vuyst, L., 2003. Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. Int. J. Food Microbiol. 81, 73–84.
Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106, 1–24.
Franz, C.M., Huch, M., Abriouel, H., Holzapfel, W., Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol. 151, 125–140.
García-Quintans, N., Repizo, G., Martin, M., Magni, C.y., López, P., 2008. Activation of the diacetyl/acetoin pathway in *Lactococcus lactis* subsp. *lactis* bv. diacetylactis CRL264 by acidic growth. Appl. Environ. Microbiol. 74, 1988–1996.
Giraffa, G., 2003. Functionality of enterococci in dairy products. Int. J. Food Microbiol. 88, 215–222.
Hennig, S., Ziebuhr, W., 2010. Characterization of the transposase encoded by IS256, the prototype of a major family of bacterial insertion sequence elements. J. Bacteriol. 192, 4153–4163.
Kempler, G., McKay, L., 1980. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Applied and environmental. Microbiology 39, 297–926.
Kim, J.H., Chambliss, G.H., 1997. Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and *amyO* target site. Nucleic Acids Res. 25, 3490–3496.
Leboeuf, C., Leblanc, L., Auffray, Y., Hartke, A., 2000. Characterization of the *ccpA* gene of *Enterococcus faecalis*: identification of starvation-inducible proteins regulated by CcpA. J. Bacteriol. 182, 5799–5806.
Lebreton, F., Willems, R.J.L., Gilmore, M.S., 2014. *Enterococcus* diversity, origins in nature, and gut colonization. In: Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N. (Eds.), *Enterococci*: From Commensals to Leading Causes of Drug Resistant Infection [Internet]. Massachusetts Eye and Ear Infirmary, Boston Available from: http://www.ncbi.nlm.nih.gov/books/NBK190427/.
Magni, C., de Felipe, F.L., Sesma, F., de Mendoza, D., 1994. Citrate transport in *Lactococcus lactis* biovar diacetylactis: expression of the plasmid-borne citrate permease P. FEMS Microbiol. Lett. 118, 75–82.
Magni, C., de Felipe, F.L., Lopez, P., de Mendoza, D., 1996. Characterization of an insertion sequence-like element identified in plasmid pCIT264 from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. FEMS Microbiol. Lett. 136, 289–295.
Magni, C., de Mendoza, D., Konings, W.N., Lolkema, J.S., 1999. Mechanism of citrate metabolism in *Lactococcus lactis*: resistance against lactate toxicity at low pH. J. Bacteriol. 181, 1451–1457.
Martin, M., Corrales, M.A., de Mendoza, D., López, P., Magni, C., 1999. Cloning and molecular characterization of the citrate utilization *citMCDEFGRP* cluster of *Leuconostoc paramesenteroides*. FEMS Microbiol. Lett. 174, 231–238.
Martin, M., Magni, C., Lopez, P., de Mendoza, D., 2000. Transcriptional control of the citrate-inducible *citMCDEFGRP* operon, encoding genes involved in citrate fermentation in *Leuconostoc paramesenteroides*. J. Bacteriol. 182, 3904–3912.

Martin, M.G., Sender, P.D., Peirú, S., de Mendoza, D., Magni, C., 2004. Acid-inducible transcription of the operon encoding the citrate lyase complex of *Lactococcus lactis* biovar diacetylactis CRL264. J. Bacteriol. 186, 5649–5660.

Martin, M., Magni, C., de Mendoza, D., Lopez, P., 2005. CitI, a transcription factor involved in regulation of citrate metabolism in lactic acid bacteria. J. Bacteriol. 187, 5146–5155.

Mortera, P., Pudlik, A., Magni, C., Alarcón, S., Lolkema, J.S., 2013. Ca^{2+}-citrate uptake and metabolism in *Lactobacillus casei* ATCC 334. Appl. Environ. Microbiol. 79, 4603–4612.

Ogier, J.C., Serror, P., 2008. Safety assessment of dairy microorganisms: the *Enterococcus* genus. Int. J. Food Microbiol. 126, 291–301.

Opsata, M., Nes, I.F., Holo, H., 2010. Class IIa bacteriocin resistance in *Enterococcus faecalis* V583: the mannose PTS operon mediates global transcriptional responses. BMC Microbiol. 25 (10), 224.

Rea, M.C., Cogan, T.M., 2003. Glucose prevents citrate metabolism by enterococci. Int. J. Food Microbiol. 88, 201–206.

Repizo, G.D., Mortera, P., Magni, C., 2011. Disruption of the *alsSD* operon of *Enterococcus faecalis* impairs growth on pyruvate at low pH. Microbiology 157, 2708–2719.

Repizo, G.D., Blancato, V.S., Mortera, P., Lolkema, J.S., Magni, C., 2013. Biochemical and genetic characterization of the *Enterococcus faecalis* oxaloacetate decarboxylase complex. Appl. Environ. Microbiol. 79, 2882–2890.

Sarantinopoulos, P., Kalantzopoulos, G., Tsakalidou, E., 2001. Citrate metabolism by *Enterococcus faecalis* FAIR-E 229. Appl. Environ. Microbiol. 67, 5482–5487.

Sarantinopoulos, P., Makras, L., Vaningelgem, F., Kalantzopoulos, G., De Vuyst, L., Tsakalidou, E., 2003. Growth and energy generation by *Enterococcus faecium* FAIR-E 198 during citrate metabolism. Int. J. Food Microbiol. 84, 197–206.

Schumacher, M., Allen, G., Diel, M., Seidel, G., Hillen, W., Brennan, R., 2004. Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. Cell 118, 731–741.

Sender, P.D., Martín, M.G., Peirú, S., Magni, C., 2004. Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family. FEBS Lett. 570, 217–222.

Sobczak, I., Lolkema, J.S., 2005. The 2-hydroxycarboxylate transporter family: physiology, structure, and mechanism. Microbiol. Mol. Biol. Rev. 69, 665–695.

Solovyev, V., Salamov, A., 2011. Automatic annotation of microbial genomes and metagenomic sequences. In: Li, R.W. (Ed.), Metagenomics and its Applications in Agriculture. Nova Science Publishers, Biomedicine and Environmental Studies, pp. 61–78.

Suárez, C.A., Blancato, V.S., Poncet, S., Deutscher, J., Magni, C., 2011. CcpA represses the expression of the divergent cit operons of *Enterococcus faecalis* through multiple *cre* sites. BMC Microbiol. 11, 227.

Suarez, C., Repizo, G., Espariz, M., Magni, C., Alarcón, S., 2012. Detection and identification of *tyrDC*+ enterococcal strains from pasteurized commercial cheeses. Food Sci. Biotechnol. 21, 603–606.

Suárez, C.A., Espariz, M., Blancato, V.S., Magni, C., 2013. Expression of the agmatine deiminase pathway in *Enterococcus faecalis* is activated by the AguR regulator and repressed by CcpA and PTS(Man) systems. PLoS ONE 8, e76170.

Vaningelgem, F., Ghijsels, V., Tsakalidou, E., De Vuyst, L., 2006. Cometabolism of citrate and glucose by *Enterococcus faecium* FAIR-E 198 in the absence of cellular growth. Appl. Environ. Microbiol. 72, 319–326.

Zuljan, F.A., Repizo, G.D., Alarcon, S.H., Magni, C., 2014. α-Acetolactate synthase of *Lactococcus lactis* contributes to pH homeostasis in acid stress conditions. Int. J. Food Microbiol. 188, 99–107.