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Original Article

Experimental evolution forcing *Oenococcus oeni* acid tolerance highlights critical role of the citrate locus

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ABSTRACT

Oenococcus oeni is the main lactic acid bacterium associated with malolactic fermentation (MLF) of wines. MLF plays an important role in determining the final quality of wines. Nevertheless, due to the stressful conditions inherent to wine and especially acidity, MLF may be delayed. This study aimed to explore by adaptive evolution improvements in the acid tolerance of starters but also to gain a better understanding of the mechanisms involved in adaptation toward acidity. Four independent populations of the *O. oeni* ATCC BAA-1163 strain were propagated (approximately 560 generations) in a temporally varying environment, consisting in a gradual pH decrease from pH 5.3 to pH 2.9. Whole genome sequence comparison of these populations revealed that more than 45% of the substituted mutations occurred in only five loci for the evolved populations. One of these five fixed mutations affects *mae*, the first gene of the citrate operon. When grown in an acidic medium supplemented with citrate, a significantly higher bacterial biomass was produced with the evolved populations compared to the parental strain. Furthermore, the evolved populations slowed down their citrate consumption at low pH without impacting malolactic performance.

1. Introduction

Malolactic fermentation (MLF) consists, *inter alia*, in the biological transformation of malic acid into lactic acid and carbon dioxide and plays an important role in determining the final quality of wines. There are three main consequences of MLF in wine: reduction in acidity, microbial stability and improvement of organoleptic qualities [1]. Usually, MLF takes place spontaneously after alcoholic fermentation but may be delayed due to harsh environmental conditions including high alcohol content, low temperature, low pH, sulfur dioxide and nutrient deficiency. Most of these factors may vary according to the climate. Cool climate wines are often more acidic than warm climate ones; as a result, malic acid concentrations may be as high as 9 g/L [2]. In cool regions, MLF is necessary to reduce total acidity. Nevertheless, at pH lower than 3.3, spontaneous MLF is unpredictable and difficulties are often encountered [3]. In such cases, starter cultures are often added for a better management of MLF, including (i) a shorter lag phase and a faster decarboxylation rate of malic acid, (ii) a reduction in potential spoilage, (iii) the control of the strain responsible for the MLF and its contribution to the wine quality. Although the inoculation of starters reduces MLF delays, its use can be challenging when the conditions are particularly harsh, resulting in the rapid loss of viability after inoculation. *Oenococcus oeni* is the most commonly used species as malolactic starter because of its capacity to overcome the stressful conditions of wine given its acid tolerance and because of the flavor profile it produces.

O. oeni shows a very high genetic and phenotypic diversity, adapted to its restricted ecological niche of fermenting fruit juices, alcoholic beverages such as wine and cider and the nonalcoholic

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beverage kombucha obtained from sugared tea [4]. Multilocus sequence typing analyses and genomic studies of *O. oeni* strains have suggested a distribution into four phylogenetic groups [5] with a correlation between product and phylogenetic group. It suggests a domestication of *O. oeni* to product but also to specific alcoholic beverages. The cellular mechanisms involved in *O. oeni* survival in oenological conditions have been widely studied in the ATCC BAA-1163 strain. Four major mechanisms were reported: (i) activation of MLF which maintains the internal pH by consuming a proton and generates a proton motive force [6], (ii) activation of the F_0F_1 ATPase that functions as a proton extruding pump [7], (iii) regulation of stress gene expression [8], and (iv) modification of membrane fluidity [9].

In addition to MLF, citrate metabolism also contributes to the generation of a proton motive force. In *Lactococcus* and *Leuconostoc* species, citrate is internalized by an electrogenic mechanism generating a membrane potential and the consumption of a proton during the decarboxylation of oxaloacetate into pyruvate which contributes to the generation of a pH gradient [10]. Citrate metabolism has been thoroughly studied in lactic acid bacteria since this organic acid is the precursor of flavor compounds that contribute to the organoleptic properties of fermented foods. Citrate utilization requires only three enzymes. After being transported inside the cell by a permease (MaeP or CitP), citrate is converted to acetate and oxaloacetate by the citrate lyase complex (CL complex and activation proteins encoded by *citCDEFXG*). Then oxaloacetate is decarboxylated by the oxaloacetate decarboxylase (Mae or CitM) yielding pyruvate and carbon dioxide. In *O. oeni* and similarly in *Weissella paramesenteroides, Leuconostoc mesenteroides* and *Lactiplantibacillus plantarum*, the genes involved in citrate utilization are organized in a typical cluster: *citR*, *mae*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG* where *citR*, encoding a transcriptional regulator, is oriented divergently with respect to all other genes. The activity of the transcriptional activator CitI (or CitR) can be modulated in response to citrate availability. As a consequence, in *W. paramesenteroides* and in *L. mesenteroides,* the transcription of the *cit* locus is induced by citrate [11,12].

In order to improve malolactic starters, a better understanding of the mechanisms and genes involved in *O. oeni* survival and growth in wine is required. Recently, a global approach by experimental evolution was performed on *O. oeni* to improve its ethanol tolerance [13]. This experiment, performed on a commercial wine strain, confirmed the possibility to outperform the *O. oeni* tolerance to high ethanol concentrations while avoiding recombinant modifications.

The aims of this study were to improve the acid tolerance of *O. oeni* using a directed evolution approach and to pinpoint candidate genes involved in acid tolerance. The ATCC BAA-1163 strain was propagated in a temporally varying environment, consisting in a gradual pH decrease from 5.3 to 2.9 over 20 months (about 560 generations). Whole genome sequencing of the evolved populations provided the opportunity to identify candidate genes involved in acid tolerance in *O. oeni*. Five loci were mutated in all evolved populations and four of them are homologous to loci known to contribute to the stress response in different bacteria, as the citrate locus. This study focused specifically on this locus and the impact of its mutation on the kinetics of citrate consumption. Benefits gained were observable by a significantly higher bacterial biomass produced with the evolved populations compared to the parental strain when bacteria were grown in an acidic medium supplemented with citrate.

2. Materials and methods

2.1. Bacterial strain, growth conditions

The *O. oeni* ATCC BAA-1163 strain isolated from Bordeaux wine was grown statically at 28 °C in FT80m (i.e. Fructose Tween 80 medium [14] modified as mentioned below) or in GEMO medium (GEMO for Growth Effective Medium for *Oenococcus oeni*). The FT80m medium contains: 5.0 g/L meat extract (instead of casamino acids), 4.0 g/L yeast extracts, 5.0 g/L glucose, 3.5 g/L fructose, 10 g/L D-L malic acid, 0,6 g/L KH_2PO_4, 0.45 g/L KCl, 0.13 g/L $CaCl_2$, 0.13 $MgSO_4$, 0.003 $MnSO_4$ and 1 mL of Tween 80. When specified, medium was supplemented with 0.3 g/L or 3.0 g/L citrate. The GEMO medium contains 2.1% concentrated white grape juice (Colindis, France), 33 g/L yeast extract, 0.08 g/L $MnSO_4$, 1 g/L KH_2PO_4, 3 g/L sodium acetate, 0.3 g/L citrate, 5 g/L L-malic acid and 0.1% Tween 80. Cell growth was performed in filled 15 mL conical centrifuge tubes (Greiner Bio-one, Austria) or in 48 well cell culture plates (Corning Incorporate, USA) using the Infinity 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland).

For stress challenges, cells were grown in FT80m pH 5.3 until the end of the exponential growth phase ($OD_{600nm} = 0.8$) and cultures were diluted in fresh FT80m medium (pH 5.3) at an OD_{600nm} of 0.2. Then, cells were harvested by centrifugation (3 min at 8, 000 g) and resuspended into FT80m pH 1.9 and incubated 60 min at 28 °C. Cultivability was determined by counting CFUs on FT80m agar plate (pH 5.3).

2.2. Experimental evolution

Four *O. oeni* populations were propagated at 28 °C over 20 months in FT80m medium. The four bacterial populations named A, B, C and D from four isolated colonies of the ATCC BAA-1163 strain were first grown in FT80m and each culture was divided into two parts. One part was replicated at pH 5.3 and served as control populations. The second part was propagated in a temporally varying environment which consisted in a gradual (but with increasing time intervals between pH changes) decrease in pH from 5.3 to 2.9 over 20 months. The two sets of cultures were passaged as soon as the culture reached the stationary growth phase by transferring 0.3 mL of each culture into 14.7 mL (1/50 ratio) of fresh FT80m medium. Final biomass and time to reach the stationary growth phase differed depending on the propagation step and the impact of acidification. Inoculation at 2% of each population produced 5.65 ($Log(N/N_0)$) generations per propagation cycle and the 560th generation was reached for the two sets of cultures.

2.3. Analytical method for monitoring organic acid consumption

Cell cultures (200 μL) were centrifuged (3 min at 8000 g) and supernatants were stored at −20 °C until further citrate and malate quantification using enzymatic kits (Biosentec, France) according to the manufacturer's instructions.

2.4. qPCR analysis

Gene expression was quantified by RT-qPCR using Real Time PCR System CFX96™ (Biorad, USA) and with specific primers designed with Primer 3® 4.1.0 software (SD1). *ldh* and *gyrA* were used as reference genes [15,16]. Cells were grown until the exponential growth phase ($OD_{600nm} = 0.8$ in GEMO pH 4.8 and $OD_{600nm} = 0.4$ in GEMO pH 3.6), harvested by centrifugation (3 min, 8000 g at 4 °C) and resuspended into 1 mL of NucleoZOL (Macherey-Nagel, France). Glass beads (0.4 g, Scientific Industries) were added to the cell samples before disrupting using Precellys® homogenizer (Precellys, France) as follows: 3 cycles of 30 s at 6500 rpm twice. Total RNAs were purified using Nucleospin RNA columns (Macherey-Nagel, France) following manufacturer's instructions.

RNA samples (2 μg) were treated by DNase I (Thermo Fisher Scientific, Germany) following manufacturer's instructions. 1 μg DNase-treated RNA was reverse transcribed using a High-Capacity

cDNA Reverse Transcription Kit (Applied Biosystems™). Real-time PCR was performed using iTaq™ Universal SYBR® Green Supermix (Biorad). Standard curves for each pair of primers were run to check qPCR efficiency. Samples of total RNA which did not undergo reverse transcription were used as controls of the absence of residual genomic DNA.

Amplifications were carried out with an initial step at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 53.5 °C for 30 s.

Results were analyzed using the comparative critical threshold (ΔCT) method as previously described [17]. Each result was obtained from the average of biological triplicates which in turn were obtained from the average of technical duplicates.

2.5. Genomic DNA isolation and illumina whole-genome (WGS) sequencing

Whole population genomic DNA was extracted using the NucleoSpin® Microbial DNA kit (Macherey-Nagel) according to the manufacturer's instructions. Sequencing libraries were prepared using NEBNext® Ultra™ II FS DNA Library Prep Kit (New England Biolabs, Beverly, MA, USA). Sequencing was performed on NextSeq500 Illumina (Illumina, San Diego, CA, USA) with 2 × 75 bp paired-end reads and a coverage >200 folds. *De novo* assembly of the ATCC BAA-1163 strain was performed using SPAdes [18]. 5,409,680 reads (227 × coverage) were assembled in 35 contigs, with an average contig length of 50,878 and an N50 of 138,471. Contigs were annotated by using the automatic annotation pipeline Prokka [19]. Mutations were identified using the computational pipeline BRESEQ [20] by aligning reads to the contigs of the ATCC BAA-1163 sequence. We used the pool mode (-p option) of BRESEQ to identify mutations present at low frequency in the evolved population. Criteria were a threshold of 2.5% mutated reads with at least 15 reads. Unmapped reads were assembled by using SPAdes [18]. Contigs were short (<1.2 kb) with low coverage (<5×) and likely due to some reads of lower quality.

3. Results

3.1. Directed evolution of O. oeni ATCC BAA-1163 until pH2.9

In order to select a more acid-tolerant *O. oeni* strain, an experimental evolution process was carried out in FT80m medium with the ATCC BAA-1163 strain. Over 20 months, four independent populations were propagated with a gradual decrease in pH from 5.3 to 2.9. As the pH decreased, the growth of bacteria slowed. Accordingly, the populations were propagated with increasing time intervals between pH changes as shown in Table 1. Simultaneously, the same four initial clones were propagated in FT80m medium at fixed pH 5.3 and these populations were used as controls. After 20 months, corresponding to over 560 generations, four evolved populations called A46FJ, B47FJ, C48FJ, D49FJ and two (of the four) control populations A43FJ, B44FJ were further analyzed.

3.2. Convergent evolution in five loci is a landmark of adaptation to low pH

Whole genome sequencing of the evolved populations A46FJ, B47FJ, C48FJ, D49FJ and the two control populations A43FJ, B44FJ was performed and mutations compared to the parental ATCC BAA-1163 genome assembly were detected using the pool option of BRESEQ [20]. In the six evolved population we identified a total of 99 mutations present in at least 10% of the reads or with a lower frequency but in loci showing other mutations at least at this rate (SD2). Remarkably, we identified 20 cases of convergent evolution in loci showing independent mutations in two or more cultures. Seven involved the two cultures grown at constant pH, nine in cultures grown with decreasing pH and only three with low penetrance involved cultures grown under both conditions. This first observation showed that even under constant condition the strain accumulate adaptive mutations and that the two conditions led to different evolutionary trajectories. Here we focused our analysis on the mutations selected under decreasing pH conditions.

Five loci were mutated in the four cultures evolved under decreasing pH conditions (Table 2). Homologs of four of these five loci have been shown to contribute to the stress response in different bacteria. First the four cultures showed the same deletion of four codons in *rpsU* encoding ribosomal protein L12. A mutation in *rpsU* (R17P) has been shown to activate the sigma B stress response in *Listeria monocytogenes* [21]. Sigma B is missing in *O. oeni*. However, the four isolates are also mutated in *ndoA*, a gene encoding a putative nuclease similar to the MazF toxin from the type 2 toxin antitoxin system MazEF. A similar toxin (66% identities) is encoded by a gene located upstream of the *sigB* locus in *L. monocytogenes* [22] and has been shown to contribute to, its stress adaptation. Therefore, these two mutations might also contribute to stress adaptation, independently of sigma B. A second regulatory pathway involving the second messenger cyclic-di-AMP is altered in the four lineages. Indeed, each populations shared mutations in the cyclic-di-AMP phosphodiesterase GdpP. In the D49FJ lineage, two different mutations were found in 89 and 9.9% of the reads respectively. The first mutation is a non-sense mutation at codon 372 likely inactivating the enzyme. We may therefore assume that the different mutations will lead to a decreased phosphodiesterase activity and therefore an increase in cyclic-di-AMP concentration (c-di-AMP). In Gram positive bacteria, c-di-AMP modulate a broad range of physiological adaptation including K^+ transport and osmotic homeostasis, which could be altered at reduced pH [23–25].

Identified mutations in the four lineages pointed also a gene encoding for a putative multidrug resistance protein MdtG belonging to the major facilitator superfamily (MFS). All characterized drug multiporters of the MFS probably function by H^+ antiport [26]. Mutations fixed in *mdtG* are nonsense mutations or

Table 1
O. oeni experimental evolution steps in decreasing pH FT80m medium.

pH medium	5.3	4.5	4.0	3.9	3.7	3.5	3.2	3.0	2.9
Bacteria generations	1st to 28th	to 58th	to 86th	to 114th	to 148th	to 182nd	to 274th	to 452nd	to 560th
Time in days[a]	3	3	3	4	4	4	5	6	14

[a] Average number of days between pH changes.

Table 2
Mutation in the five loci affected in the four evolved lineages.

Gene, Function	Mutation, Mutation effect	Evolved lineages[a]			
		A46FJ	B47FJ	C48FJ	D49FJ
rpsU, 30S ribosomal protein S21	Δ12 bp,[b](163–174/195 nt)	100%	100%	100%	100%
ndoA, Endoribonuclease EndoA	G→A, G10R				85.7%
	G→T, Q25H			100%	
	G→T, L32F		100%		
	Δ1 bp,[b](219/357 nt)	100%			
gdpP, Cyclic-di-AMP phosphodiesterase GdpP	G→T, S494Y			100%	
	T→C, Q490R	100%			
	+CAA,[b](1333/2058 nt)		100%		
	G→A, R372[d]				89.0%
mdtG, Multidrug resistance protein-like MFS transporter	G→T, S228[d]	100%			
	G→C, S152[d]				89.6%
	Δ1 bp,[b](356/1230 nt)			100%	
	Δ19 bp,[b](207–225/1230 nt)				7.2%
	C→T, V27I			100%	
	+AT,[b](51/1230 nt)		100%		
cydD/acmC/citR/mae, *Membrane transporter CydD, N-acetylmuramidase AcmC, Transcriptional regulator CitR, Oxaloacetate decarboxylase Mae*	Δ3,610 bp,[b](558^{cydD}/703ma nt)				100%
citR ←/→ ***mae***	T→A, intergenic[c](-125/-25)			100%	
citR ←/→ ***mae***	G→T, intergenic[c](-142/-8)		100%		
Mae	C→A, S48[d]	100%			

[a] Percentage of mutated reads.
[b] Position in the coding sequence.
[c] Position with respect of the start codon of the two genes.
[d] Means stop codon.

frameshift mutations which are predicted to inactivate this system and prevent the H^+ internalization in the evolved populations. In addition, lineage C48FJ and D49FJ were mutated for a putative magnesium transporter of the CorA family (SD2). Globally, reducing membrane permeability to cation could be under positive selection.

Finally, several mutations affected metabolic functions and more specifically citrate metabolism (Table 2 and SD2). Lineage C48FJ is mutated in *alsS* encoding the acetolactate synthase converting pyruvate to acetolactate, whereas lineages A46FJ and D49FJ are mutated in *ackA* encoding the acetate kinase converting acetyl-P to acetate along with the phosphorylation of ADP. However, the most striking observation was the presence of different mutations affecting the *mae* locus for citrate degradation fixed in the four populations evolved at low pH. Judging from their positions (Fig. 5), the mutations substituted in the *mae* gene in the four lineages A46FJ, B47FJ, C48FJ and D49FJ may all have an impact on protein synthesis. The mutation fixed in the A46FJ population is a nonsense mutation modifying the 48th codon (Ser → STOP). Mutations substituted in the B47FJ and C48FJ lineages were located in the *citR-mae* intergenic region. Regarding the position of the mutations and the nucleic acid sequence, we suggest that the fixed mutation in B47FJ disrupted the RBS and that the fixed mutation in C48FJ was located within the −10 region of the *mae* promotor. The D49FJ population contained a large deletion of 3610 pb including *citR*, the putative regulator of the citrate operon, the entire intergenic region and a part of the *mae* gene. Therefore, the appearance of fixed mutations which could impact the *mae* expression in all the evolved populations suggests a potential role of this gene and of citrate metabolism in acid sensitivity. As *mae* is the first gene of the citrate operon, mutations which occurred in the regulatory region may also affect the expression of the entire regulon, and strongly impact citrate metabolism.

3.3. Improved growth for the evolved populations in pH 3 medium supplemented with citrate

To estimate the benefits gained by directed evolution under decreasing pH conditions, the acid resistance of the four evolved populations A46FJ, B47FJ, C48FJ and D49FJ was first evaluated by measuring their survival to a stress challenge (60 min at 28 °C in FT80m, pH 1.9) (Fig. 1). Unexpectedly, the evolved populations A46FJ, B47FJ and C48FJ were significantly more sensitive (more than 1 log) or as sensitive (D49FJ) to this acid challenge than the parental strain. Therefore, these harsh conditions were likely not adapted to estimate the benefits gained by the evolved populations.

To highlight a gain acquired by the evolved populations and because of fixed mutations targeting the citrate locus, we focused our analyses on citrate metabolism. The growth of B47FJ and D49FJ populations was compared to the growth of the parental strain in FT80m medium supplemented or not with citrate (Fig. 2 and Table 3). Two citrate concentrations were tested: 0.3 g/L which corresponds to the amount that can be found in wine and 3 g/L which is closer to the conditions tested by Augagneur *et* al. [27]. At pH 5.3, the optimal pH value for the growth of *O. oeni* in FT80m medium, the growth rates of the parental and the evolved populations did not show any significant differences, whatever the citrate concentration. Nevertheless, in the presence of 3.0 g/L of citrate, the biomass produced at the end of the growth was significantly weaker for the evolved population compared to the parental strain (delta of 0.210 and 0.271 ODU in B47FJ and D49FJ, respectively). This difference may reflect the impact of accumulated mutations during the evolution process on the growth in primordial conditions, *i.e.* in pH 5.3 FT80m medium. Nevertheless, at pH 5.3, the combination of the two organic acids, citrate and malate, still led to a higher yield in all strains, in agreement with previous reports [27–29]. However, this stimulating effect was not observed

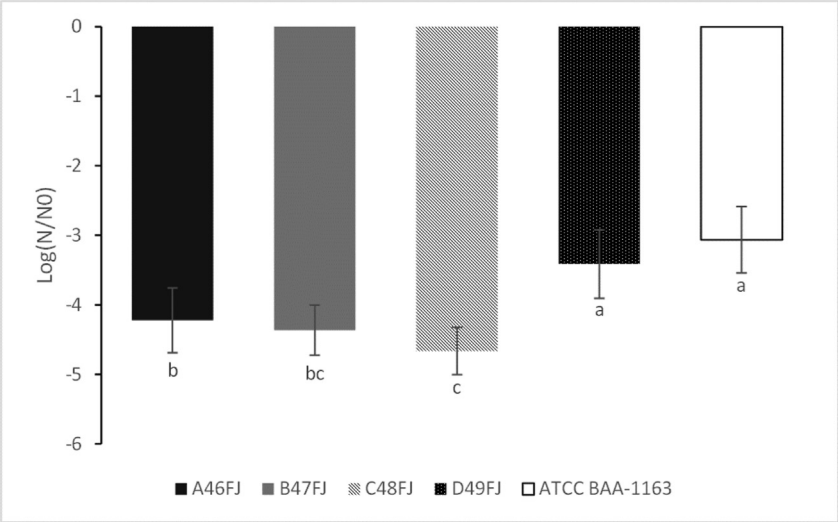

Fig. 1. Cultivability decrease in evolved and parental strains after a drastic acid challenge. The evolved populations A46FJ, B47FJ, C48FJ, D49FJ and the parental strain ATCC BAA-1163 were grown in FT80m medium (pH 5.3) at 28 °C until mid-exponential growth phase, cultures were normalized to $OD_{600nm} = 0.2$ and directly transferred into FT80m adjusted to pH 1.9. Cultivability was estimated after 60 min at 28 °C by enumerating CFU/mL on FT80m agar plates. The decrease in cultivability was expressed as Log(N/No). Error bars correspond to the confidence interval with $\langle = 0.05$ ($n = 3$). Significant differences are based on the Kruskal–Wallis test ($p < 0.05$).

at pH 3. On the contrary, citrate seems to have a deleterious effect on the growth of *O. oeni*, as previously highlighted by Augagneur et al. [27]. Indeed, at pH 3, the highest growth yield was obtained in medium without citrate, with a final OD of between 0.411 and 0.501 according to the population. No significant difference in the final OD of the three populations was observed under these conditions and the growth rate was also similar for all strains. However, the addition of citrate in this acid medium led to a sharp decrease in the final biomass produced by the parental strain (reaching 72% decline with 3.0 g/L citrate). A more moderate decline was noted in evolved populations B47FJ and D49FJ (12% and 18% decline, respectively, with 3.0 g/L citrate). Similarly, the growth rate dropped by more than half in the parental strain in the presence of 3.0 g/L of citrate (61% decline); whereas a more modest decline was observed in the evolved populations B47FJ (44% decline) and D49FJ (only 10% decline). We therefore propose, in accordance with previous reports, that citrate induces strong growth inhibition of the ATCC BAA-1163 strain under acidic conditions. This effect is significantly tempered in the evolved populations, and particularly in the D49FJ population, with a smaller decrease in the growth rate, maintaining more than 82% of the biomass formed in the presence of both organic acids, citrate and malate, at low pH. Thus, growth monitoring in medium with malate and citrate revealed the benefits acquired by evolved populations B47FJ and D49FJ in acidic conditions. Other mutations in the evolved populations might also contribute to this phenotype, particularly the *ackA* mutation in the D49FJ population. Indeed, some bacteria like *Lactococcus lactis* subsp. *cremoris* [30] possess more than one gene encoding an acetate kinase, whereas *O. oeni* has only one. Thus, the *ackA* mutation could also reduce acetate production in D49FJ population, which substantially impaired the *O. oeni* growth under low pH.

3.4. Slowdown in citrate consumption in the evolved populations

In order to evaluate the capacity of the evolved populations to conduct MLF, growth and organic acid consumption in the two evolved populations B47FJ and D49FJ were compared to the performance of the ATCC BAA-1163 parental strain in a white grape juice medium (GEMO medium) containing citrate and L-malate. This growth medium was selected to reach a higher growth rate for *O. oeni* under low pH conditions than in the FT80m medium and to perform growth monitoring in a medium with a composition closer to that of wine. Experiments were conducted at pH 4.8 (Fig. 3), which is the optimal value for the growth of the parental strain in GEMO medium and at pH 3.6 (Fig. 4) which enables a sufficient growth of the bacteria while approaching the pH of wine.

The parental strain and the evolved populations B47FJ and D49FJ presented similar growth rates (μmax = 0.194, 0.183 and 0.184 h^{-1}, respectively) at pH 4.8 (Fig. 3A). All populations presented an identical malate consumption kinetics with a utilization rate of around 1.5 g of malate consumed per generation (Fig. 3C). Therefore, no difference in MLF performance was observed and malate was totally metabolized after 20 h. In contrast, citrate consumption profiles were different among populations (Fig. 3B). Consumption was delayed in the evolved populations, starting after half of the malate was consumed, and more pronouncedly delayed in the D49FJ population which began to metabolize citrate after a lag time of 20 h, at which time the malate was exhausted (Fig. 3B). The citrate-consumption rate was also significantly impacted in evolved populations B47FJ and D49FJ, which metabolized 0.077 g and 0.041 g of citrate per generation respectively. In comparison, the parental strain consumed 0.083 g per generation with a co-utilization of malate and citrate. Citrate metabolism is profoundly affected in the evolved populations, with a slowdown in the consumption kinetics and a reduced efficiency in the utilization of citrate.

At pH 3.6, a significant difference in growth rates was observed among cultures of the three strains (Fig. 4D) although the growth kinetics remained similar (Fig. 4A). Citrate consumption onset in the evolved populations occurred much later than at optimal pH, especially in the D49FJ population which had consumed no more than one third of the citrate after 56 h compared to all the citrate for the parental strain in 16 h (Fig. 4C). However, the growth rate was greater at pH 4.8 than at pH 3.6 and the parental strain consumed

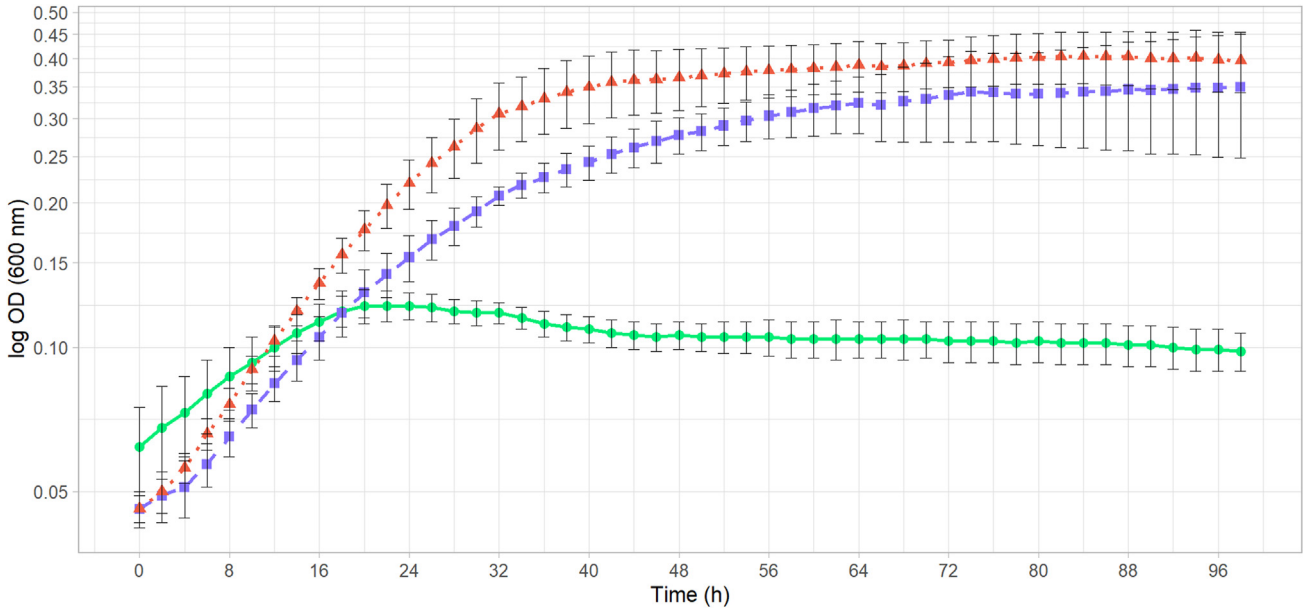

Fig. 2. Influence of pH and citrate on the growth of the parental strain and evolved populations. The growth of the ATCC BAA-1163 parental strain (solid green lines, round points) and of evolved populations B47FJ (dashed violet lines, square points) and D49FJ (dotted red lines, triangle points) was monitored at 28 °C in pH 3.0 FT80m medium supplemented with 3.0 g/L citrate. Values are the means of three independent experiments with ±SD.

Table 3
Influence of pH and citrate on the growth of evolved populations in FT80m medium.

			ATCC BAA-1163		B47FJ		D49FJ	
	[citrate] g/L		[a]μ_{max}	[b]Final OD	μ_{max}	Final OD	μ_{max}	Final OD
pH 5.3		0	0.159 ± 0.035	1.002 ± 0.020	0.156 ± 0.025	1.010 ± 0.022	0.151 ± 0.018	1.086[c] ± 0.002
		0.3	0.159 ± 0.027	1.177 ± 0.033	0.174 ± 0.031	1.158 ± 0.014	0.167 ± 0.033	1.179 ± 0.019
		3.0	0.160 ± 0.026	1.437 ± 0.027	0.198 ± 0.013	1.277[c] ± 0.022	0.189 ± 0.020	1.166[c] ± 0.012
pH 3.0	0		0.123 ± 0.016	0.44 ± 0.012	0.118 ± 0.027	0.411 ± 0.086	0.083 ± 0.015	0.501 ± 0.094
	0.3		0.08 ± 0.010	0.299 ± 0.036	0.066 ± 0.003	0.408 ± 0.081	0.075 ± 0.006	0.585[c] ± 0.005
	3.0		0.047 ± 0.013	0.122 ± 0.009	0.066 ± 0.008	0.362[c] ± 0.090	0.074[c] ± 0.002	0.408[c] ± 0.048

Values are the means of three independent experiments with ±SD.
[a] μmax (h^{-1}) was determined during the exponential growth phase.
[b] Final OD corresponds to the optical density at 600 nm reached at the stationary growth phase.
[c] Means significant differences from the ATTCC BAA-1163 ancestral strain, based on Kruskal–Wallis test ($p < 0.05$).

citrate faster at low pH (0.138 g of citrate per generation at pH 3.6 *versus* 0.083 g citrate per generation at pH 4.8). In contrast, the utilization rate of citrate was even slower at pH 3.6 in population D49FJ (0.016 g citrate per generation at pH 3.6 *versus* 0.041 g of citrate per generation at pH 4.8). Population B47FJ consumed citrate at pH 3.6 as fast as at pH 4.8 (0.079 g per generation at pH 3.6 *versus* 0.077 g per generation at pH 4.8). These modifications in citrate utilization observed in the evolved populations demonstrated that the mutations in the citrate locus have a strong impact on this organic acid metabolism, even though the utilization of citrate was not fully impaired.

These experiments, conducted at pH 4.8 (Fig. 3B and E) and pH 3.6 (Fig. 4B and E), did not reveal any variations in the kinetics nor in the malate consumption rate (around 1.6 g of malate per generation) when comparing the parental strain and the evolved populations. The increase in the malate consumption rate observed in D49FJ (2.1 g per generation) may be linked to the slower growth rate of this evolved population. No enhanced ability to conduct MLF on the part of the evolved populations was thus brought to light under the tested experimental conditions; the slowdown in citrate metabolism could nonetheless represent an interesting point for industrial applications.

3.5. Evolved populations showed a drastic reduction of the citrate locus expression

In order to further understand the differences observed in citrate consumption kinetics among strains, RT-qPCR analysis was performed to quantify gene expression during growth (Fig. 5). Prior to this, PCR amplifications were performed on cDNA (data not shown), confirming that *mae* and *maeP* as well as *maeP* and *citC* are co-transcribed as previously described in other genetically close lactic acid bacteria belonging to the *Leuconostoc* genus [11,12,31].

The RT-qPCR analysis (Fig. 5) revealed significant differences in the citrate gene expression when comparing the parental strain and the two evolved populations, B47FJ and D49FJ. While the

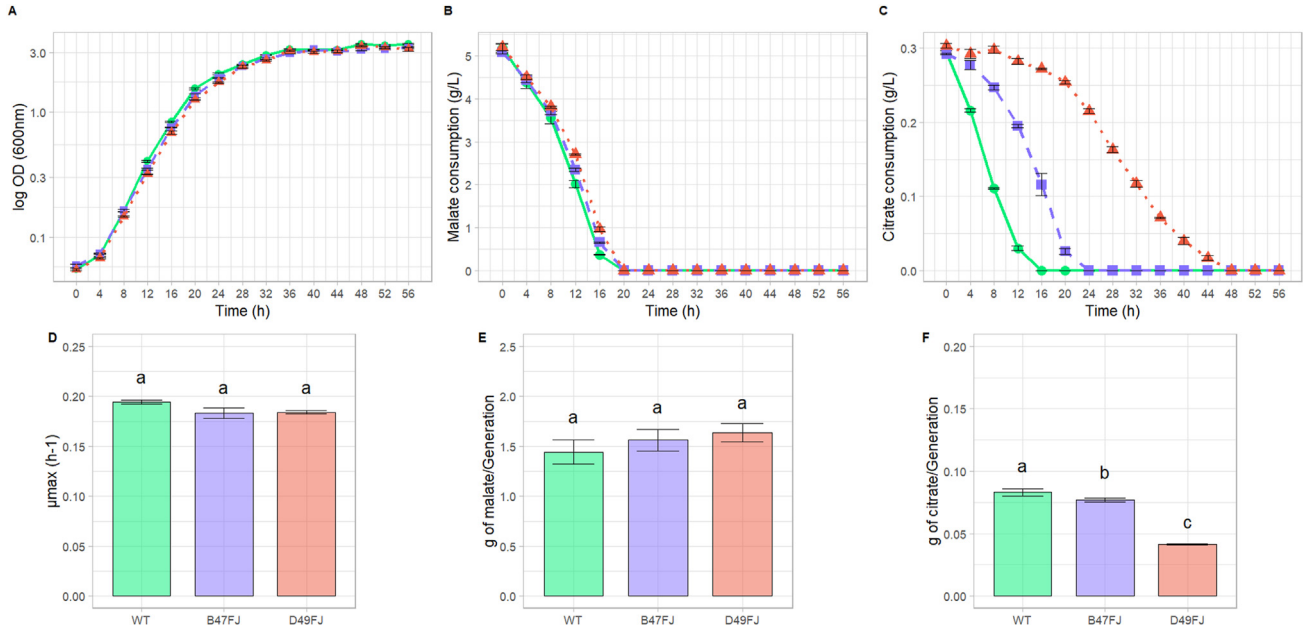

Fig. 3. Organic acid consumption during growth in pH 4.8 GEMO medium. The growth of the ATCC BAA-1163 parental strain (solid green lines, round points) and of evolved populations B47FJ (dashed violet lines, square points) and D49FJ (dotted red lines, triangle points) was monitored at 28 °C in pH 4.8 GEMO medium (A) as was malate (B) and citrate consumption (C). The growth rates (D) and the organic acids consumed by generation for malate (E) and citrate (F) are represented under each curve. Data represent means of three, independent biological replicates. Significant differences are based on the Kruskal–Wallis test ($p < 0.05$).

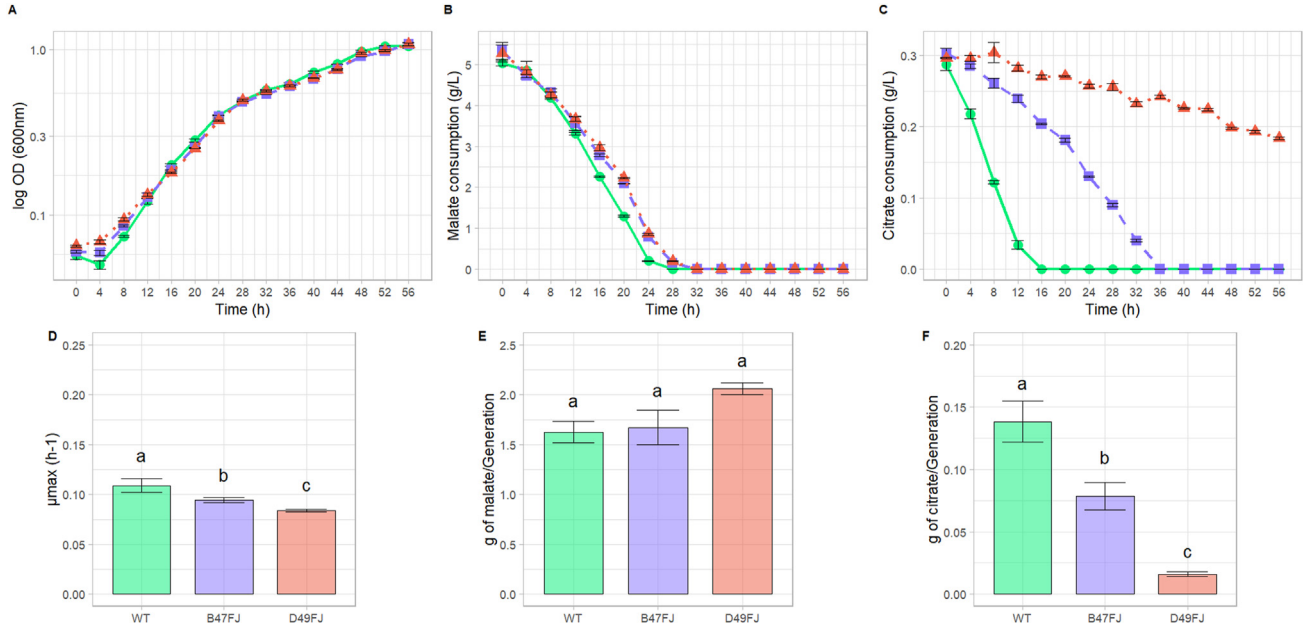

Fig. 4. Organic acid consumption during growth in pH 3.6 GEMO medium. The growth of the ATCC BAA-1163 parental strain (solid green lines, round points) and of the evolved populations B47FJ (dashed violet lines, square points) and D49FJ (dotted red lines, triangle points) was monitored at 28 °C in pH 3.6 GEMO medium (A) as was malate (B) and citrate consumption (C). The growth rate (D) and organic acids consumed by generation for malate (E) and citrate (F) are represented under each curve. Data represent means of three, independent biological replicates. Significant differences are based on the Kruskal–Wallis test ($p < 0.05$).

expression of *citR* did not seem to be impacted in the B47FJ population compared to the parental strain, all other citrate genes underwent a drastic reduction of their expression in the evolved populations. The *mae* gene expression decreased by more than 10-fold in population B47FJ for both pH conditions. *maeP and citE* expression levels showed more moderate reductions, about 4-fold, compared to the parental strain. Regarding the D49FJ population, which sustained a 3 kb-deletion including the *citR* and *mae* genes, the expression of the *maeP* and *citE* genes was hardly detected, whatever the pH, compared to the parental strain. No *citR* or *mae*

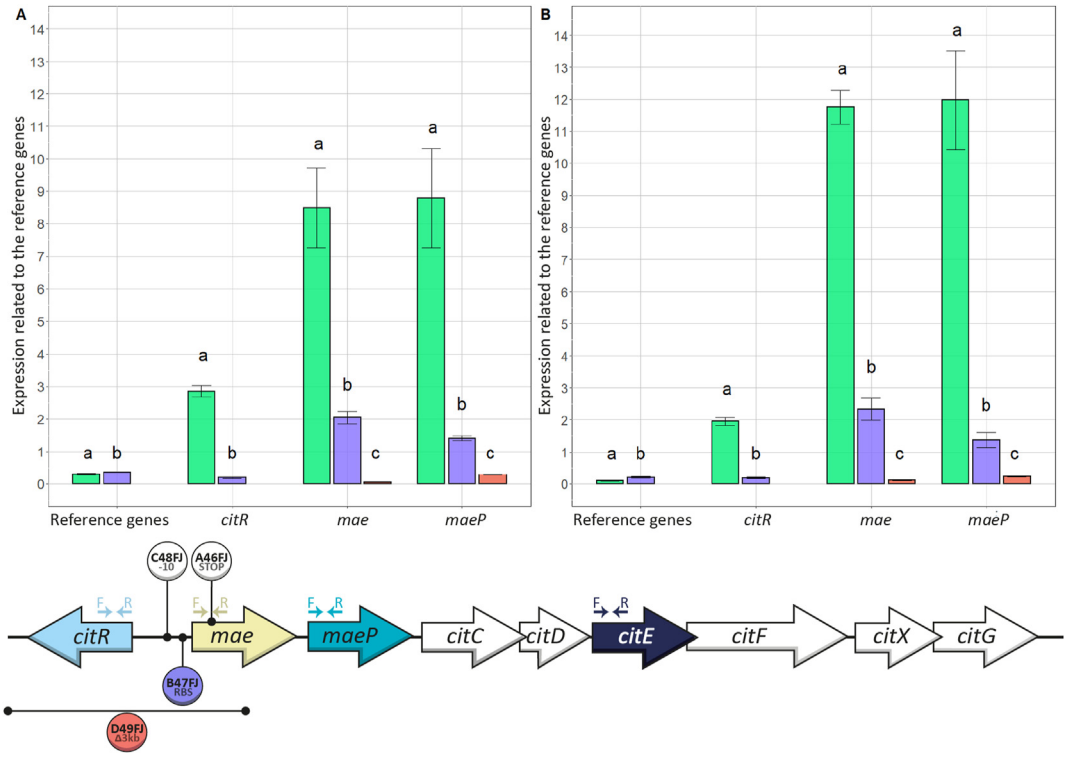

Fig. 5. Comparison of the relative expression level of genes involved in citrate metabolism between the parental strain and the evolved populations. The parental ATCC BAA-1163 strain (green), B47FJ (violet) and D49FJ (red) populations were grown in pH 4.8 GEMO medium (A) or pH 3.6 GEMO medium (B). RNA was extracted from cells harvested at the mid-exponential growth phase (OD_{600nm} = 0.8 and 0.4 respectively for growth in pH 4.8 and pH 3.6 medium). The expression level of the citrate genes was related to the expression of the two reference genes *ldh* and *gyrA*. Pair of primers (arrows F and R) used for qPCR analysis are shown on the physical map of the *O. oeni* citrate locus (on the bottom). The position of mutations substituted in the *mae* gene is indicated for A46FJ, B47FJ and C48FJ populations and the horizontal line indicates the large deletion (Δ 3610 pb) of D49FJ population. Results were obtained from biological triplicates run twice in the same plate (technical duplicates). Significant differences are based on the Kruskal–Wallis test ($p < 0.05$).

messenger was detected upholding the chromosomal deletion. These transcriptional results are in agreement with the data monitoring citrate consumption.

4. Discussion

The current study allows to identify candidate genes involved in acid tolerance in *O. oeni*. In recent years, directed experimental evolution on *O. oeni* to produce more robust strains have been published [13,32], demonstrating the feasibility of improving starters by means of directed evolution. However, the evolved population were not genome sequenced and hence mutations contributing to adaptation not identified. Here, we analyzed for the first time genotypic and phenotypic changes following the laboratory evolution of *O. oeni* under acidic selective pressure. Genome comparison of evolved populations reveals that fixed mutations were commonly observed in five loci of the four lineages propagated in decreasing pH medium. These loci are known to be involved in stress response in other microorganisms, so they could be regarded as candidate beneficial mutation for acid tolerance. Mutations in *gdpP* are attractive for future research as *gdpP* mutations have been shown to confer resistance to acid, high temperature, cell wall stressors suggesting a general stress resistance mechanism [24,33]. *gdpP* disruption increases intracellular c-di-AMP level, a nucleotide signaling molecule which has been implicated in the control of several facets of bacterial physiology [34]. Alterations of c-di-AMP homeostasis affects multiples cell functions, including overall bacterial fitness. However, response to pH changes mediated by c-di-AMP is species specific. Indeed, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Lactococcus lactis* and *Bacillus subtilis* phosphodiesterase-deficient mutants are more resistant to acidic conditions than their parental strains whereas *Streptococcus pneumoniae gdpP* mutants are highly susceptible to low pH. Likewise, mutations in the *ndoA* gene encoding a putative nuclease similar to the MazF are attractive. MazEF is one of the most studied toxin antitoxin systems in bacteria and MazF homologs have important physiological roles in stress adaptation and drug tolerance. This site-specific mRNA interferase is also essential for programmed cell death [35].

Sequential batch culture was deemed appropriate to control the progressive decrease in pH in the medium according to the slow bacterial growth. The FT80m medium, the one used in our laboratory since the 1980's to undertake *O. oeni* ATCC BAA-1163 strain growth, was chosen to avoid adaptive mutation events linked to medium composition. Unexpectedly, a harsh acid challenge performed in the FT80m pH 1.9 medium revealed that after approximately 560 generations the three independent evolved populations A46FJ, B47FJ and C48FJ were significantly more sensitive than the parental strain ATCC BAA-1163 (Fig. 1). This revealed that acid tolerance needs to be differentiated from resistance. A parallel can be made here with antibiotic resistance *versus* tolerance. Typically, antibiotic resistance reduces the effectiveness of the drug concentration whereas tolerance increases resilience to antibiotic treatment duration in a nonspecific way [36]. Thus, acid tolerance would mean that the bacteria can grow and undergo normal functions despite the acidity of the medium whereas resistance, tested by a drastic acid challenge, may concern other transitional molecular mechanisms allowing the cell to deal with harsh and quick

stressing conditions. Given that this drastic acid challenge did highlight a potential gain by the evolved populations, complementary investigations were undertaken.

Here we focused our analyses on the impact of mutations of the *mae* gene encoding a putative oxaloacetate decarboxylase on the assumption that it is implicated in citrate metabolism, the precursor of diacetyl. Citrate, as well as malate, contribute to pH homeostasis by generating a proton motive force in *O. oeni* [6,27,37]. Thus, the appearance of fixed mutations targeting the *mae* gene in the four evolved lineages was unexpected. However, a negative impact of citrate on *O. oeni* growth at pH 3.2 has been shown by Augagneur et al. [27], in contradiction with the positive effect of this organic acid reported on *Leuconostoc* and *Lactococcus* growth [38,39]. This negative impact on *O. oeni* growth at low pH did not significantly affect the maintenance of the pH gradient and the authors suggested that one of the citrate metabolism end products, such as acetate, could inhibit growth, as the addition of acetate substantially impaired the growth rate of the bacterium. Our results confirmed a stimulating effect of citrate on the growth of the parental strain at pH 5.3 (Fig. 1B). Conversely, its growth is strongly inhibited at pH 3 by the addition of citrate, in agreement with Augagneur et al. [27]. The evolved populations B47FJ and D49FJ were significantly less impacted by the presence of citrate at pH 3, with a biomass produced and a growth rate significantly higher than the parental strain (Fig.2 and Table 3). On the other hand, the citrate stimulating effect at pH 5.3 medium is reduced in both evolved populations compared to their common ancestor. These results highlight the positive selection of the directed evolution experiment on growth abilities under these drastic conditions. In a medium closer to oenological conditions, a slight decline in the growth rate of the evolved populations was observed (Fig. 4D), but without a significant difference in the final biomass produced. Moreover, a complementary experiment conducted in pH3.2 GEMO medium demonstrated an increase in the final biomass produced by the two evolved populations (13−18% increase, respectively) compared to the parental strain (0.760 *versus* 0.875 and 0.920 for the B47FJ and D49FJ, respectively). This point reveals that the performance of the strains differs depending on the matrix, and that the evolved populations acquired beneficial mutations enhancing acid tolerance in a highly acidic medium including citrate.

Monitoring of organic acid consumption did not show differences in either the kinetics or the malate consumption rate when comparing the parental strain and evolved populations, whatever the pH of the medium (Figs. 3 and 4). The consumption of L-malate stimulates the growth and contributes to pH homeostasis by generating a proton motive force in *O. oeni* [6]. This pathway involves *mleA* and *mleP* genes encoding the malolactic enzyme (MLE) and malate permease, respectively. No fixed mutations could be noted in the *mle* locus of the evolved populations, confirming the importance of maintaining this pathway.

The parental strain showed a concomitant degradation of citrate and malate independently of the pH of the medium. However, it appears that citrate consumption in the evolved populations is slowed down and that the lower the pH of the medium, the more pronounced this slowdown. The citrate metabolism of population D49FJ, which carries a large deletion in the 3′-region of the citrate operon (3610 pb including *citR* and a part of *mae*), achieved the biggest slowdown. This clearly showed that a shunt of the citrate consumption was made possible by means of a directed evolution approach.

Transcriptional analysis of the citrate operon supports the fact that *mae*, *maeP* and *citC* are co-transcribed in the *O. oeni* ATCC BAA-1163 strain as reported for other genetically related species [31]. In *Weissella paramesenteroides*, the citrate cluster is transcribed in two divergently messengers: *citMCDEFGRP* and *citI* [12]. The *citI* gene, similar to *citR* of *O. oeni*, encodes a transcriptional activator whose activity can be modulated in response to citrate availability [12,31]. In *O. oeni* two consensus CitR binding operators are conserved in the *citR-mae* intergenic region, suggesting a similar mechanism of transcriptional regulation. The quantification of *mae* transcripts revealed a lower relative expression level of this gene compared to the expression of *maeP* and *citE* in the parental strain as well as in population B47FJ, whatever the pH value (Fig. 5). These findings suggest a probable post-transcriptional regulation with a specific cleavage of a large polycistronic transcript to ensure a suitable proportion of each enzyme implicated in citrate metabolism and potentially to uncouple the expression of the *mae* gene from those of other *cit* operon genes, as previously suggested in reference to *W. paramesenteroides* [12]. This regulation may ensure that there is no accumulation of potentially toxic citrate intermediate products, such as oxaloacetate or pyruvate. Another explanation may be the presence of a transcription start site downstream of *mae*. That assumption is supported by the detection of *maeP* and *citE* transcripts in the D49FJ evolved population despite a large deletion including *citR* and over half of *mae*. Nevertheless, complementary experiments need to be undertaken to localize transcriptional start sites and to determine a probable post-transcriptional regulation.

The reduced citrate metabolism observed in the evolved populations is in agreement with the decrease in *mae, maeP* and *citE* transcript levels. The comparison between citrate metabolism and citrate gene expression in the B47FJ and D49FJ populations demonstrated that the lower the citrate locus expression, the slower the citrate metabolism. In evolved population B47FJ, the mutation fixed on the potential RBS upstream of the *mae* gene could disfavor the interaction of the mRNA with the ribosome and consequently impact its translation and stability. Likewise, this mutation could affect the interaction of a transcriptional activator, like CitI (CitR) described in *W. paramesenteroides* [31] on the assumption that *maeP* and *citE* transcript levels are also drastically decreased in this evolved population. Future experiments will focus on the molecular function of the *O. oeni* CitR.

To conclude, the present study demonstrates that experimental evolution conducted in an increasingly acidic environment *via* a sequential batch system opens up exploratory paths concerning *O. oeni* gene functions and points to promising candidate genes in further understanding the mechanisms of acid tolerance in this bacterium and to improve it. There are, however, a variety of important criteria to consider when selecting malolactic starters, including the ability to tolerate high ethanol and sulfite concentrations. More in-depth analysis of the behavior of evolved populations is on-going to evaluate strain performance in the wine environment with multiple related stresses. Commonly used starters have different genetic potentials for the buttery aroma. Random mutagenesis has previously been used to disrupt citrate consumption in a commercial *O oeni* strain (Viniflora®CiNE™, Ch. Hansen, Denmark). This citrate non-fermenting *O. oeni* strain, carrying a nonsense mutation in the citrate transporter gene enables winemakers to perform MLF in wines without producing buttery flavors or sacrificing the fresh and fruity characters. This phenotype strain comes in response to the increased consumer demand for fresh and fruity red wine to be drunk young [40]. By means of the present work, we show that similar phenotype strains can be obtained by a direct evolution approach. This is a point of interest for companies whose aim is to develop industrial starters characterized by low diacetyl production.

Author contributions

CG, SG, CR, FJ and CE designed the study. FJ performed the experimental evolution of the ATCC BAA-1163 strain. NC performed genome sequencing and run analytical program. PG performed genome analyses and characterized mutations and contribute to writing of the manuscript. FJ and NT performed stress challenge. CE performed monitoring organic acid consumptions and transcriptomic analysis. HA, JC and MF provided critical feedback of the manuscript. CG took the lead in the writing of this article and all authors analyzed data, contributed to manuscript revision, and approved the submitted version.

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Declaration of competing interest

The authors declare that this work was conducted in the absence of any known potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2023.104048.

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