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Investigating *Oenococcus oeni* citrate locus expression: Role of *citR* and impact on acid tolerance

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ABSTRACT

A previous adaptive evolution experiment performed on the wine bacterium *Oenococcus oeni* revealed the beneficial effect of slowing down citrate metabolism on the acid tolerance of this bacterium. This finding prompted us to investigate the regulation pathways of the *cit* locus expression in this lactic acid bacterium, an investigation which has never been undertaken before. Mapping the *cit* locus, composed of a long *mae-maeP-citCDEFXG* cluster preceded by *citR* in a divergent direction, highlighted the expression of a *citR* messenger overlapping the coding sequence of *mae*, the first gene of the operon. Transcriptional analysis of the locus suggested that the transcript of *maeP*, encoding the citrate permease, could be stabilize by this antisense. Furthermore, genetic complementation has demonstrated the transcriptional regulatory function of CitR on the *cit* operon expression. This work contributes to a new understanding of the regulation of citrate metabolism in *O. oeni* which is involved in diacetyl production giving a buttery aroma to wine. It also provides new evidence for the involvement of citrate metabolism in the acid stress response of this bacterium, therefore in its ability to conduct malolactic fermentation in very acid wines.

1. Introduction

Oenoccocus oeni is the main lactic acid bacterium (LAB) responsible for malolactic fermentation (MLF) in wine. This fermentation converts L-malic acid (a major grape organic acid) into L-lactic acid and CO_2 (Volschenk et al., 2006). The desired effect of MLF on wine is a decrease in total acidity, influencing the sensory profile of wine by decreasing sourness (Bartowsky, 2005). In addition, the consumption of the remaining carbon sources as well as the production of several aromatic compounds by the bacteria prevent the development of spoilage microorganisms and enhance the organoleptic properties of wines (Kunkee, 1991; Bauer and Dicks, 2004; Lerm et al., 2010). Nevertheless, wine is a difficult environment for microorganisms to colonize, mainly because of the presence of ethanol, the conditions of low pH and low temperature, the lack of nutrients and the presence of sulfites (Versari et al., 1999; Lerm et al., 2010; Bech-Terkilsen et al., 2020). Commercial starters are sometimes used to ensure MLF despite the difficult environmental conditions. This solution is not always sufficient and wine-related stresses can result in stuck or sluggish fermentations. For these reasons, improved knowledge of the mechanisms involved in the resistance of *O. oeni* to various wine-related stresses is necessary.

Bacterial response mechanisms to acid stress have raised particular interest in recent years (Margalef-Català et al., 2016; Liu et al., 2017; Yang et al., 2019; Qi et al., 2021; Chen et al., 2022). To highlight unknown mechanisms implied in the acid stress response of *O. oeni*, an experimental evolution was performed on the ATCC BAA-1163 strain to adapt it to low pH (Julliat et al., 2023). In the course of this experiment, bacteria acquired mutations that became fixed in the genome of the evolved populations. The citrate locus, enabling citrate uptake and metabolism, was part of the gene targeted by fixed mutations. Citrate is an organic acid naturally present in grape musts at a concentration ranging from 0.13 to 0.90 g/L (Alexandre et al., 2008). The fact that *O. oeni* is able to metabolize citrate is an important parameter to consider during the winemaking process, as this metabolism leads to the

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production of diacetyl, a C4-compound. Diacetyl lends buttery notes to wine that are undesirable beyond a certain threshold (Bartowsky and Henschke, 2004). It appears that the mutations acquired by evolved populations in the *cit* locus trigger a slowdown in citrate metabolism which seems to be correlated with better acid tolerance (Julliat et al., 2023).

The citrate operon in *O. oeni* is composed of 8 genes *mae-maeP-citCDEFXG*: *maeP* encodes a uniport belonging to the metabolite/H^+ symporter family for the internalization of the anionic form of citrate (Ramos et al., 1994); *citD*, *E* and *F* encode the three subunits γ, β, α of citrate lyase responsible for the conversion of citrate into acetate plus oxaloacetate which is activated by the products of *citC*, *X* and *G* genes (Bekal et al., 1998; García-Quintáns et al., 2008); finally, *mae* encodes a putative oxaloacetate decarboxylase for the conversion of oxaloacetate to pyruvate (Sender et al., 2004; García-Quintáns et al., 2008). Upstream of this operon and in the divergent direction, *citR* encodes a putative transcriptional regulator belonging to the SorC family. Due to the lack of suitable genetic tools, no study has yet been carried out to explore the *citR* function in *O. oeni*. CitR shares 52 % of its identity with the CitI of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis and 38 % with CitI from *Weissella paramesenteroides* (Eicher et al., 2024a). The activator function of CitI on the transcription of the *citMCDEFGRP* operon of *W. paramesenteroides* was previously demonstrated (Martín et al., 2000; Martin et al., 2005). CitI interacts with two operator sites localized in the intergenic region upstream of *citM*, the homolog to *mae*. The authors of the study have suggested that this regulation system of *cit* locus expression could be extended to all other LAB, including *O. oeni* (Martin et al., 2005).

The aim of the present study was to identify the pathways of regulation of *cit* locus expression in *O. oeni*, with a focus on the role of CitR. The transcriptional sites of the *cit* locus were mapped and the locus expression was monitored in strains with a different genetic background. The results, backed up by genetic complementation, allowed us to demonstrate the involvement of CitR in the regulation of *cit* locus expression in *O. oeni* as well as its significant impact on the acid tolerance of this bacterium.

2. Material and method

2.1. Bacterial strains, growth conditions and monitoring

O. oeni strains ATCC BAA-1163 (accession number AAUV00000000), isolated from Bordeaux red wine (France), PSU-1 (accession number CP000411), isolated from a red wine made in Pennsylvania (USA) and NB_1858 (accession number OR754048), isolated by IFV (Institut Français de la Vigne et du vin, France) were used in this study.

O. oeni was grown at 28 °C in GEMO medium (Grandvalet-Eicher Medium for *Oenococcus oeni* (Julliat et al., 2023)) pH adjusted to 4.8 or 3.0. Medium was supplemented with erythromycin (20 mg/L), vancomycin (20 mg/L) and lincomycin (20 mg/L) when necessary. Growth was performed in 48 well cell culture plates (Corning Incorporate, USA) using the Infinity 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland) or in 50 mL conical centrifuge tubes (Greiner Bio-one, Austria) in which organic acids consumption monitoring or RNA extraction was carried out. Analytical method for monitoring citrate (ref: 12825) or malate (ref: 12803) consumptions was performed on the supernatant of bacterial cultures using the automatic sequential analyzer Y15 (Biosystems, Spain).

Escherichia coli C2992 (New England Biolabs, USA), used as host strain for cloning and plasmid maintenance, was grown at 37 °C in Luria-Bertani (LB) medium agitated and supplemented with erythromycin (250 mg/L) when necessary.

2.2. RNA extraction and qPCR analysis

Total RNA extraction, DNase treatment and reverse transcription were performed as previously described (Julliat et al., 2023). cDNA obtained after reverse transcription were quantified by qPCR using Real Time PCR System CFX96™ (Biorad, USA) using SsoAdvanced Universal SYBR Green Supermix (Biorad, USA) following manufacturer's instructions. Amplifications were carried out with an initial step at 98 °C for 3 min followed by 40 cycles of 98 °C for 10 s and 53.5 °C for 30 s. Specific primers were designed with Primer 3® 4.1.0 software (Table S1). *ldh* and *gyrA* were used as the housekeeping genes (Desroche et al., 2005). 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. Results were analyzed using ΔCt method (Livak and Schmittgen, 2001; Riedel et al., 2014). Each result was obtained from the average of biological triplicates which in turn were obtained from the average of technical duplicates.

2.3. Mapping of transcription start sites

5′-RACE technique (Rapid Amplification of cDNA-Ends) was employed to localize the transcription start sites (Matteau and Rodrigue, 2015; Tillett et al., 2000). To do so, the Template Switching RT Enzyme Mix was used (New England Biolabs, USA) following the manufacturer's instructions. After reverse transcription, PCR was undertaken using Q5 Hot Start High-Fidelity Master Mix (New England Biolabs, USA). Forward primer TSO-F, annealing to the Template Switching Oligo added to the 5′-end of each transcript, and the reverse primers mea-R, maeP-R and citR-F2/SC2b-F were used to search for the transcription start sites before *mae*, *maeP* and *citR*, respectively (Table S1). PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and sequenced by Sanger sequencing (Genewiz, Germany). The sequences (Fig. S1) were aligned and compared using Clustal Omega program version 1.2.4 (Sievers and Higgins, 2021).

2.4. Prediction of mRNA and protein structures

Secondary structure of *O. oeni* CitR protein as well as the putative DNA binding sites were predicted from the amino-acid sequence of PSU-1 (accession number NC_008528 locus tag OEOE_0417) and NB_1858 (accession number OR754048) using PredictProtein online software (Qiu et al., 2020). 3D structure of the protein was found in AlphaFold 2 databases.

Secondary structures of mRNA were predicted from the DNA sequence of ATCC BAA-1163 (accession number AAUV00000000) using RNAfold WebServer (Gruber et al., 2008, 2015).

2.5. DNA manipulation and bacterial transformation

Genomic DNA, plasmid DNA and PCR DNA fragments were purified using NucleoSpin Microbial DNA kit, NucleoSpin Plasmid kit and NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), respectively. All enzymes required for cloning: GoTaq® DNA polymerase (Promega, France), Expand High Fidelity PCR System (Roche, Meylan, France), *Eco*RI and *Hind*III restriction enzymes (New England Biolabs, USA), T4 DNA ligase (ThermoScientific, USA), were used according to the protocol provided by the manufacturer.

The pSIPSYN vector was used for the complementation of the ATCC BAA-1163 strain as follows (Fig. S2). The *citR* gene, including the coding sequence and the *citR-mae* intergenic region, was amplified using Expand High Fidelity PCR System (Roche, Meylan, France). Genomic DNA of PSU-1 or NB_1858 were used as a template. The primers CitRPSU-F and pSIPCitR-R (Table S1), generating *Eco*RI and *Hind*III restriction sites at the 5′ and 3′ end of *citR*, were employed to amplify *citR*. Both 1109 bp PCR products were inserted between *Eco*RI and *Hind*III sites of pSIPSYN positioning *citR* under the control of the synthetic pSYN promoter of pSIPSYN instead of *gusA*. The resulting plasmids were named pSIPcitR-PSU and pSIPcitR-NB, respectively. The control pSIPc

vector, corresponding to pSIPSYN free of *gusA*, was constructed by *Eco*RI restriction of pSIPSYN then ligation with T4 DNA ligase (Thermo-Scientific, USA). *E. coli* C2992 was used as a host strain for cloning, then recombinant vectors were transferred in *O. oeni* after controlling nucleotide sequences of DNA fragments inserted (Sanger sequencing by Genewiz). *E. coli* and *O. oeni* were transformed by electroporation (Taketo, 1988; Assad-García et al., 2008). For *E. coli*, positive clones were selected on LB plates supplemented with erythromycin (250 mg/L) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (40 mg/L, Euromedex, France). For *O. oeni*, positive clones were selected on FT80m plates supplemented with erythromycin, vancomycin and lincomycin (20 mg/L each). Colony-PCR amplification using GoTaq® DNA polymerase (Promega, France) with primers citRend-F and gusAtermHind (Table S1) was used to check the recombinant clones.

3. Results

3.1. Transcriptional mapping of the cit locus

This study aimed to characterize citrate locus expression in *O. oeni* as well as its transcriptional regulation modes. Overlapping PCRs between all the genes of the *cit* operon were first performed on the total mRNA of ATCC BAA-1163 (see Table S1 for primers used) and these regions were successfully amplified, meaning that the whole operon is co-transcribed. The 5′-RACE technique (Rapid Amplification of cDNA Ends) was then employed to locate the transcription start sites in the *cit* locus (Matteau and Rodrigue, 2015; Tillett et al., 2000). The experiment was carried out on mRNAs extracted from ATCC BAA-1163 and PSU-1. In both strains, a transcription start site was identified 16 bp before the *mae* start codon (Fig. 1A and Fig. S1) and characteristic −10 and −35 elements could be identified upstream of this start site spaced by 17 nucleotides. This promoter may control the transcription of the entire *mae-maeP-cit-CDEFXG* cluster.

No transcription start sites could be identified for *citR* in the *citR-mae* intergenic region. Therefore, the search for +1 continued further along the operon. A start site was found within the *mae* coding sequence, preceded by typical −10 and −35 elements (Fig. 1A). As a result, the *citR* transcript overlaps 437 pb of the *cit* operon messenger, forming an antisense targeting *mae* mRNA. In addition, other 5′ extremities which are not followed by −10 and −35 sequence features were detected (Fig. 1B). This finding suggests that these sites are not effective transcription start sites, but rather the result of cleavage by RNase activity. One such site is located on *maeP*, in which a typical hairpin secondary structure was detected that could be the target of endonucleases, as

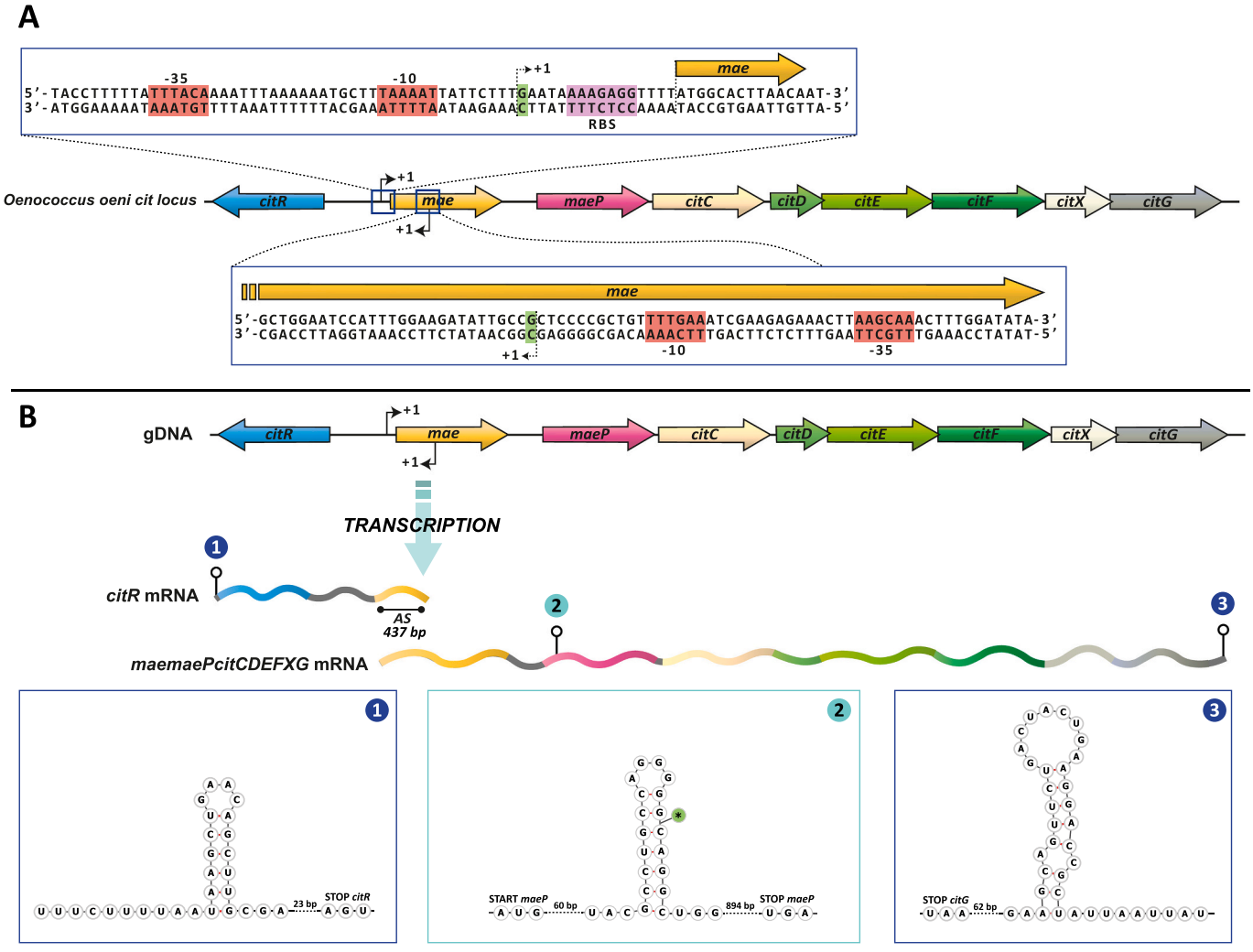


Fig. 1. Mapping of *O. oeni cit* locus. A: The identified transcription start sites are represented by nucleotides highlighted in green on the ATCC BAA-1163 *cit* locus sequence. −10 and −35 sequences are highlighted in red, while the RBS of *mae* is in pink. B: *cit* locus transcripts are represented (*citR* and *mae-maeP-citCDEFXG*). The two putative Rho-independent terminators are represented by air-pin structures as is the secondary structure on *maeP* that could be the target of endonucleases. The potential cleavage site in the *maeP* transcript detected by the 5′-Race technique is represented by a green star on the sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reported previously (de Felipe et al., 1994; Martín et al., 2000; Drider et al., 2004; Blancato et al., 2008).

3.2. Exploration of cit locus expression

To understand the regulation of *cit* locus expression, the transcript levels from *citR*, *mae*, *maeP* and *citE* were quantified by RT-qPCR during growth. This experiment was carried out on three *O. oeni* strains: (i) PSU-1 which served as a reference; (ii) ATCC BAA-1163 which possesses a nonsense mutation (L256*) in *citR*, meaning that the protein produced is non-functional in this strain; and (iii) NB_1858 which, compared to 30 other *O. oeni* strains, possesses a genetic variability (I7T) that may affect the activity of the protein (Eicher et al., 2024b) (Fig. 2A). As shown in Fig. 2B, these strains consumed citrate at different rates, with ATCC BAA-1163 consuming all citrate in 16 h while PSU-1 and NB_1858 consumed it significantly more slowly (in 32 and 36 h, respectively). Total RNA was thus extracted from these strains after 8 and 24 h of growth, corresponding to different stages of citrate consumption (Fig. 2C and D). Huge differences in transcript levels of *cit* locus genes were highlighted among the three strains. Indeed, in correlation with the fast consumption of citrate (Fig. 2B), a greater number of transcripts were quantified for the four genes monitored in ATCC BAA-1163. For example, fold changes of 4.71 and 4.21 were calculated for *mae* in ATCC BAA-1163 *versus* 0.55 and 1.26 in PSU-1 depending on the growth time. On the contrary, *cit* locus seems to be globally less expressed in the NB_1858 strain with fold changes of 0.04 to 0.17 for the *mae* gene depending on growth time.

3.3. Investigation of CitR function through genetic complementation of ATCC BAA-1163

The results reported above demonstrated a huge difference in levels of expression of citrate genes among the three strains studied. The differences found could be correlated with the genetic variability of *citR* in ATCC BAA-1163 and NB_1858. To further explore the role of CitR in *O. oeni*, the ATCC BAA-1163 strain was complemented using the replicative pSIPSYN vector (Darsonval et al., 2015, 2018) in which the *citR* gene from PSU-1 (pSIPcitR-PSU) or from NB_1858 (pSIPcitR-NB) was cloned. Recombinant vectors were transferred into the ATCC BAA-1163 strain, producing OocitR-PSU and OocitR-NB strains, respectively. The Ooc strain carrying the pSIPc was used as control (see Material and methods). Citrate consumption was monitored along with growth for each mutant (Fig. 3A and B). The monitoring of growth at pH 4.8 did not reveal any differences between strains, as a μmax of 0.2 and a maximum OD of 3.4 on average was calculated for each clone. On the other hand, the monitoring of citrate consumption revealed a slowdown in the

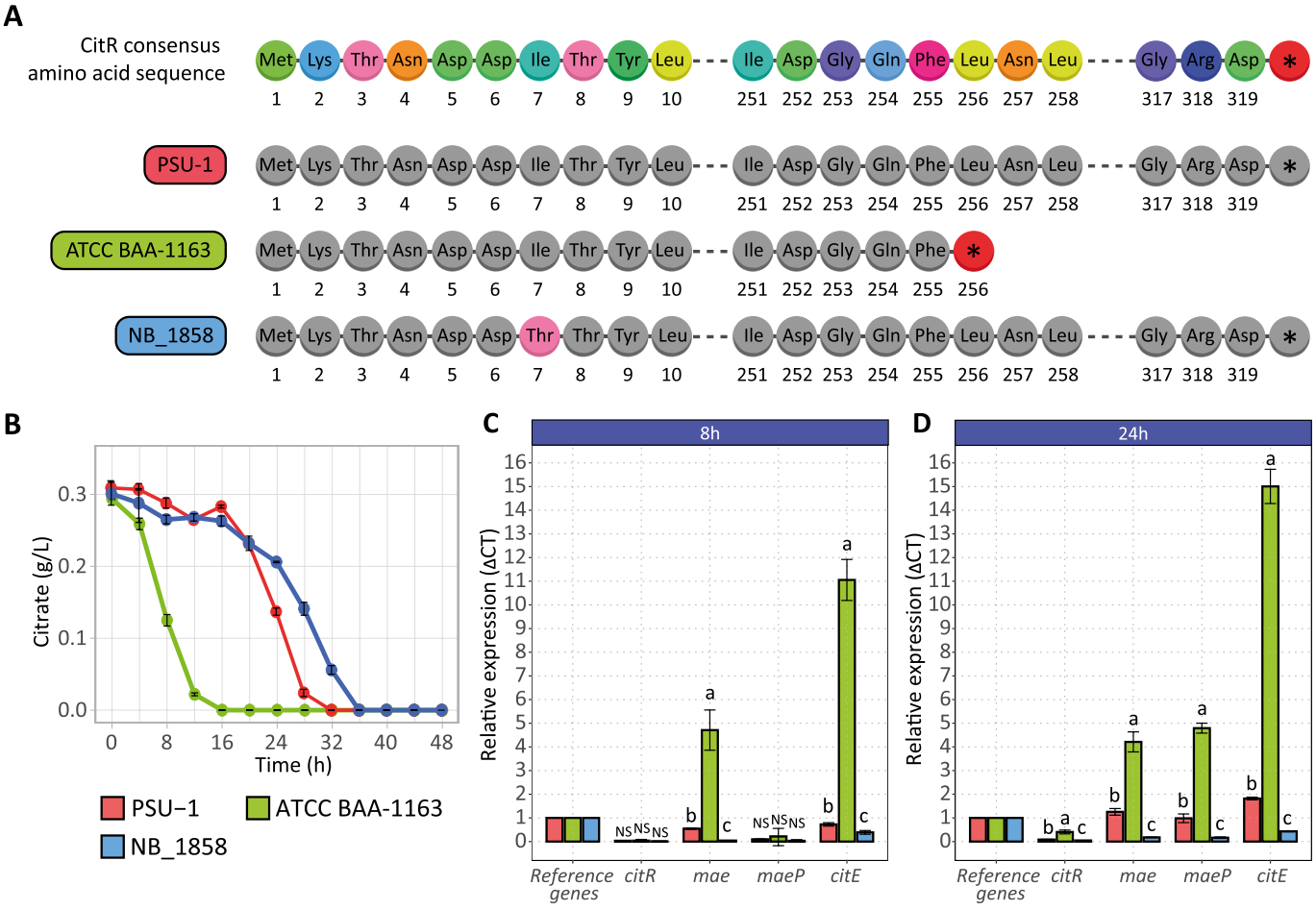


Fig. 2. Comparison of citrate consumption and *cit* locus expression over time among genetic variants. (A). Alignment of the CitR amino acid sequence from ATCC BAA-1163 (green), PSU-1 (red) and NB_1858 (blue) pinpointing the genetic variation. The CitR consensus sequence was defined thanks to the alignment of 30 *citR* sequences from different strains (Eicher et al., 2024b). (B) Citrate consumption of strains growing in pH 4.8 GEMO medium. (C and D) Expression levels of the citrate genes related to the expression of the two housekeeping genes *ldh* and *gyrA* (ΔCt method). Total RNA was extracted after 8 h (C) and 24 h (D) of growth for RT-qPCR analysis. The results were obtained from biological triplicates (n = 3) run twice on the same plate (technical duplicates). Significant differences are based on the Kruskal-Wallis test ($p < 0.05$, NS = not significant). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

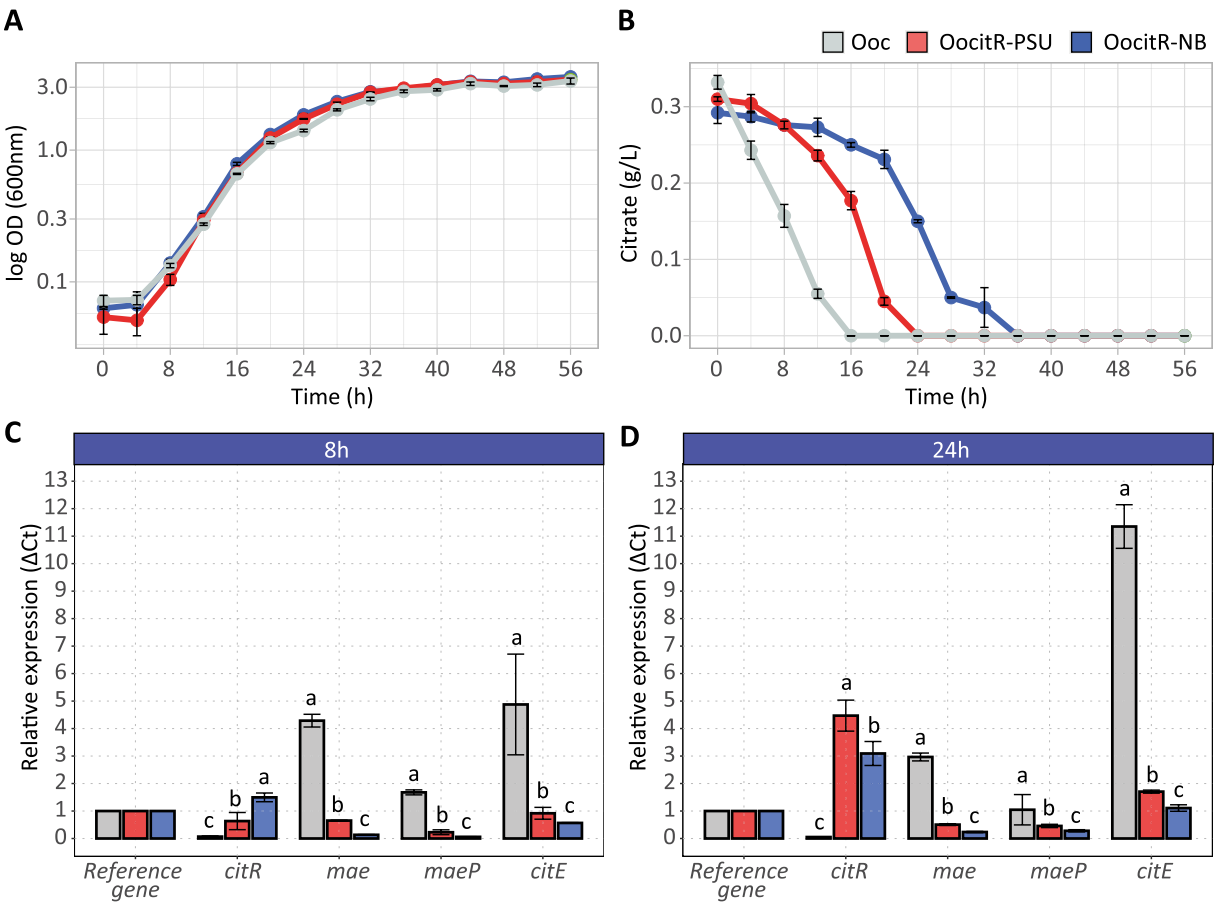


Fig. 3. The CitR complementation of ATCC BAA-1163 slowed down its citrate consumption kinetics. The *O. oeni* strains Ooc (grey), OocitR-PSU (red) and OocitR-NB (blue) were grown in pH 4.8 GEMO medium. Growth (A) occurring in pH 4.8 GEMO medium and citrate consumption (B) were monitored every 4 h. Total RNA was extracted after 8 h (C), and 24 h (D) of growth for qPCR analysis. The expression levels of the citrate genes were related to the expression of the two housekeeping genes *ldh* and *gyrA* (ΔCt method). The results were obtained from biological triplicates (n = 3) run twice on the same plate (technical duplicates). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

citrate consumption rate in the two complemented strains compared to the control, and the effect was more pronounced in OocitR-NB (Fig. 3B).

qPCR analysis performed at 8 h and 24 h using the ΔCt method (Fig. 3C and D) showed that *citR* is expressed 10 to 90 times more in the two complemented mutants than in Ooc, depending on the conditions. Accordingly, a significantly lower level of *mae*, *maeP* and *citE* transcripts could be quantified in OocitR-PSU and OocitR-NB in both conditions. Furthermore, the levels of the three genes tested were lower in the OocitR-NB strain compared to the OocitR-PSU strain, a finding which correlates with the citrate consumption rate obtained (Fig. 3B). These results comforted the hypothesis that *citR* is implied in the negative transcriptional regulation of the citrate operon in *O. oeni* and showed that *citR* from NB_1858 has a stronger effect on the repression of the *cit* operon than that of PSU-1.

3.4. Slowdown in citrate consumption triggered by genetic complementation enhances ATCC BAA-1163 acid tolerance

The experimental evolution performed on ATCC BAA-1163 suggested a link between the slowdown in citrate consumption and improved acid tolerance (Julliat et al., 2023). To verify this link, the growth of the complemented strains in pH 3.0 GEMO medium was thus monitored (Fig. 4). Similar to findings in evolved populations, the maximum OD reached in the two complemented strains was significantly higher than that of the control (OD = 0.298 and 0.415 for OocitR-PSU and OocitR-NB respectively *vs.* OD = 0.188 for the control, Fig. 4C). This complementation experiment thus confirmed that in these growth conditions, a slowdown in citrate consumption in the bacterium led to greater ability to grow at low pH.

4. Discussion

4.1. O. oeni CitR acts as a transcriptional repressor on cit locus expression

The monitoring of citrate metabolism in three *O. oeni* strains highlighted huge differences among them, correlated with the *cit* locus expression (Fig. 2B, C and D). In contrast to the reference strain PSU-1, the other two strains carry SNP in *citR* (Fig. 2A): ATCC BAA-1163 having a nonsense mutation and NB_1858 possessing a I7T substitution (Eicher et al., 2024b). Isoleucine is a hydrophobic amino acid, while threonine is hydrophilic. This amino acid may be directly involved in DNA binding interaction, and its substitution could therefore modulate the affinity of CitR for DNA (Sutton and Kaguni, 1997). Alternatively, this substitution could also impact the three-dimensional structure of the protein and, consequently, DNA interaction. To get an idea of the impact of this genetic variation on CitR, the PredictProtein tool was employed to predict the putative DNA binding sites of the protein (Fig. S3). It showed that the RI-Score (protein-ligand binding energy production (Nguyen et al., 2017)) calculated for three predicted DNA binding sites in CitR is higher in the NB_1858 sequence, the one modified by the I7T substitution, than that of PSU-1. It is therefore possible that the increase in DNA interaction sites predicted in the CitR from NB_1858 results in a stronger interaction of this protein with DNA compared to the regulator of other strains.

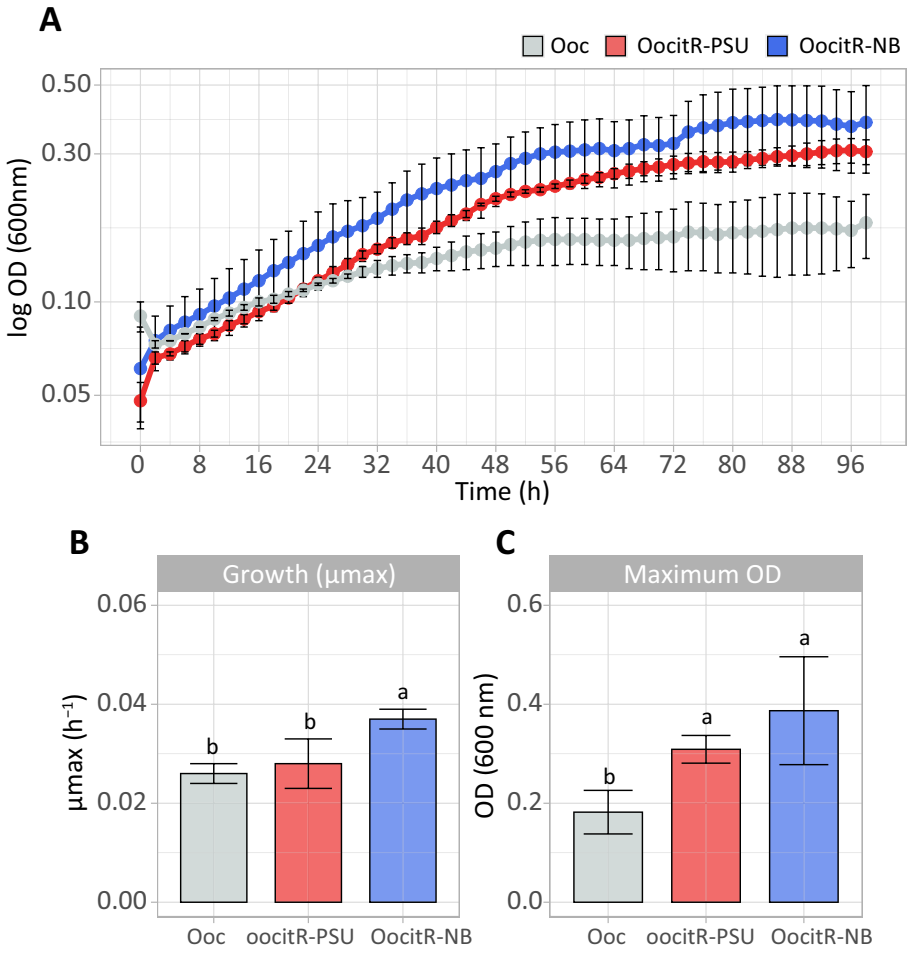


Fig. 4. CitR complementation of ATCC BAA-1163 strain enhances ability to grow at low pH. The growth of OocitR-PSU, OocitR-NB and the Ooc control strain were monitored in pH 3.0 GEMO medium containing citrate (A). The growth rate (B) and the maximum OD achieved (C) were calculated for each strain. Data represent means of three independent biological replicates (n = 3). Significant differences are based on the Kruskal-Wallis test ($p < 0.05$).

Regarding the qPCR results (Fig. 2C and D), it appeared that the *cit* mRNA level is higher in ATCC BAA-1163, possessing a non-functional CitR, than in NB_1858, whose CitR may interact more strongly with DNA. Thus, the *O. oeni* CitR regulator must act as a transcriptional repressor on the *mae-maeP-citCDEFXG* operon (Fig. 5). This conclusion was reinforced by the complementation of ATCC BAA-1163 with a functional *citR* gene which triggers the down-regulation of the *cit* operon expression (Fig. 3). Moreover, the effect was more pronounced in *citR* from NB_1858, supporting the view that its protein may more strongly repress the transcription of the *cit* locus than that of other strains. The role of CitR in *O. oeni* contrasts with what has been demonstrated in other LAB (Martín et al., 2000, 2004; Martin et al., 2005; Blancato et al., 2008). For now, the CitR regulation mechanism remains imperfectly understood, but it may be possible that, such as in other LAB, it binds its operator sites in the intergenic region before *mae* to modulate the locus expression (Martin et al., 2005; Blancato et al., 2008). Indeed, CitR amino acid sequence is close to that of CitI from *L. lactis* bv. diacetylactis (52 % of homology) (Eicher et al., 2024a). In this bacterium, two putative operator sites for CitI were identified (Martin et al., 2005). Comparing the *cit* locus sequence of several LAB including *O. oeni*, the authors found similar operator sequences that could be the operator sites of CitR. Further investigations will have to be undertaken such as DNase footprinting or Electrophoretic Mobility Shift Assay (EMSA) experiments to confirm the operator sites of CitR.

4.2. Modulation of cit locus expression improves the bacterium acid tolerance

An experimental evolution previously carried out on ATCC BAA-1163 to enhance its acid tolerance led to the fixation of mutations in the *cit* locus that triggered the slowing down of citrate metabolism (Julliat et al., 2023). This study highlighted that the slowing down of the citrate consumption rate could be responsible for better acid tolerance in *O. oeni*. Furthermore, another study carried out on this specific strain demonstrated that the presence of citrate at low pH was toxic for the bacterium (Augagneur et al., 2007). In the present study, the complementation of ATCC BAA-1163 with a functional CitR confirmed the link between citrate consumption rate and growth capacity at low pH (Fig. 4). Citrate metabolism therefore clearly plays a non-negligible role in the acid tolerance of *O. oeni*. It has been suggested that the observed toxic effect of citrate metabolism may be due to the production of acetate, a weak acid that could easily re-enter the cells at low pH (Augagneur et al., 2007). A negative effect on *L. lactis* viability has also been demonstrated when pyruvate is accumulated due to a mutation in the *alsS* gene, preventing its conversion to α-acetolactate (Zuljan et al., 2014). Measurement of intracellular pH, or metabolic by-products quantity (acetate and pyruvate) could help to verify these hypotheses. In addition, slowing citrate metabolism could induce pyruvate rerouting within the cell to facilitate cofactor reoxidation, thereby promoting growth (Eicher et al., 2024b). Labeling citrate with ^{13}C is another possibility that could highlight rerouting of citrate depending on the environmental conditions. Furthermore, the *mae* gene of the *cit* locus,

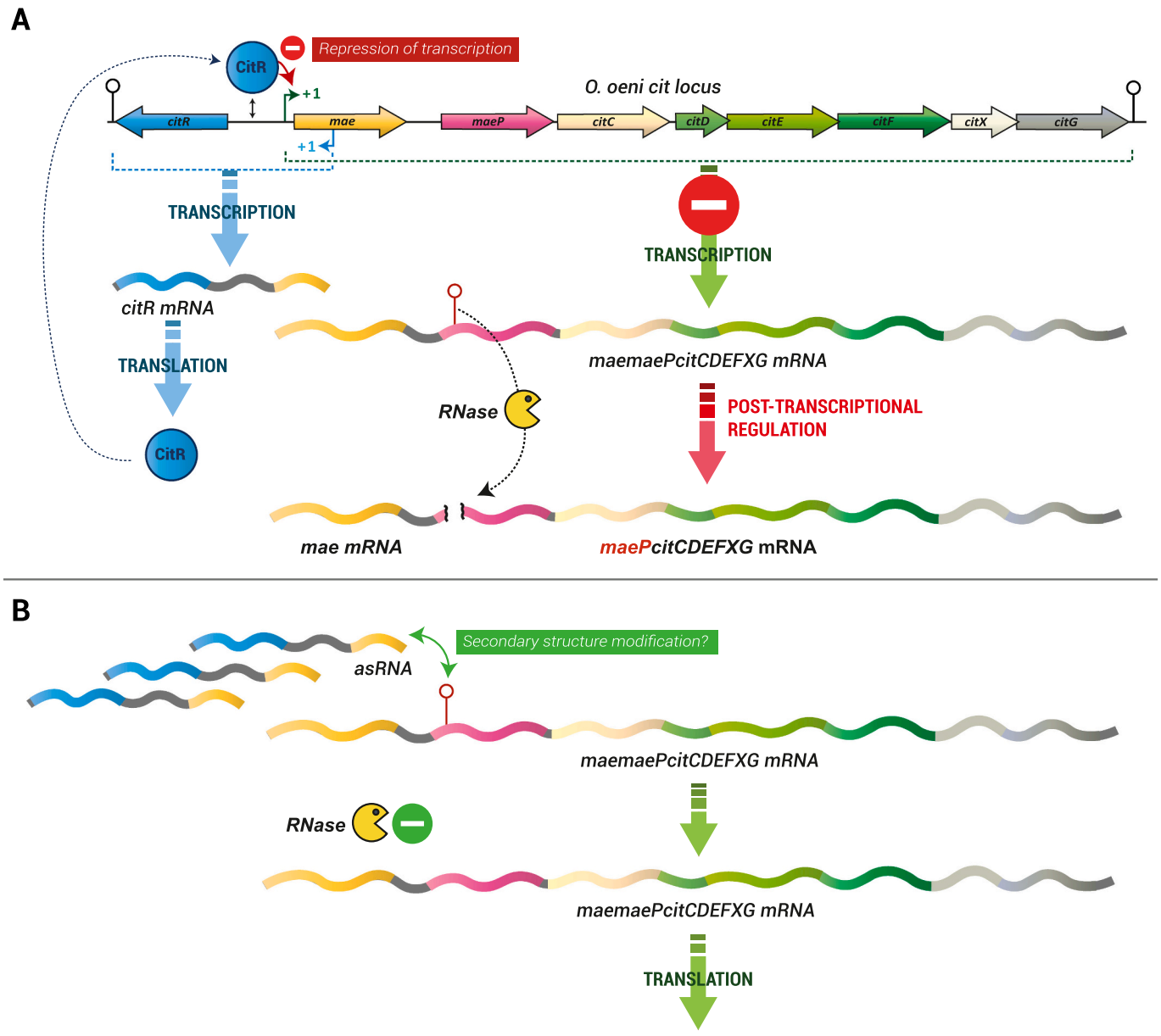


Fig. 5. Possible paths of *cit* locus expression regulation in *O. oeni*. Two transcription start sites were identified, leading to the transcription of *citR* mRNA (along with an *mae* asRNA) and *mae-maeP-citCDEFXG* mRNA. *Cit* locus expression could be regulated in two different ways: (A = negatively) CitR must be a transcriptional repressor of *mae-maeP-citCDEFXG* cluster (symbolized by red minus). A secondary structure on *maeP* mRNA (symbolized by an air-pin structure) could be the target of endonucleases preventing translation of the citrate permease; (B = positively) the annealing of *mae* asRNA to its target may modify the secondary structure of the *maeP* transcript preventing the air-pin structure formation and therefore its recognition by endonucleases (symbolized by a green minus), which could be at the origin of citrate consumption initiation in *O. oeni*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

encoding an oxaloacetate decarboxylase, is also implied in malate metabolism. The decarboxylation of L-malate to L-lactate is part of the early acid stress response of LAB and the main energy source for *O. oeni* at low pH (Beltramo et al., 2006; Contreras et al., 2018; Chen et al., 2022; Huan et al., 2023). Downregulation of *mae* may positively influence acid tolerance of *O. oeni* by preventing the conversion of L-malate to pyruvate which would cause redox imbalance. Consequently, the L-malate conversion to L-lactate would be favored (Eicher et al., 2024b). However, decreasing *mae* expression by complementing ATCC BAA-1163 with a functional *ciR* gene did not result in changes in malate consumption kinetics (Fig. S4). Monitoring the becoming of L-malate through C^{13}-labelling could be an interesting approach to verify this hypothesis.

In the light of these results, it seems logical to assume that NB_1858 would perform best at low pH, while ATCC BAA-1163 would be the least acid-tolerant. In reality, a growth monitoring carried out using the same culture conditions but at low pH showed a greater growth capacity of ATCC BAA-1163 compared with NB_1858 (Eicher et al., 2024b). In fact, it is difficult to compare these two strains since they belong to two different phylogroups and have completely different metabolic behavior. It is not just citrate metabolism kinetic that differs between these two strains. Therefore, attributing the better acid tolerance of ATCC BAA-1163 to its higher citrate consumption rate is complicated. Furthermore, citrate metabolism is singular in this strain as it consumes citrate much faster than do many other *O. oeni* strains (Eicher et al., 2024b), must probably because of the non-functionality of CitR. In view of the results obtained after complementing ATCC BAA-1163 with a functional *citR* gene, this rapid consumption of citrate could be detrimental to the cell at low pH. Therefore, the fixation of mutations in the *cit* locus observed during the experimental evolution carried out by

Julliat et al. (2023) could have occurred to restore a basal citrate consumption rate in this specific bacterium, leading to a better acid tolerance.

Furthermore, it is important to notify that comparing the *cit* locus of 30 *O. oeni* strains has shown the presence of mutations inducing a slowing down of citrate metabolism in some strains known to be highly acid-tolerant (Eicher et al., 2024b). This suggests that a slowing down of citrate metabolism is indeed beneficial at low pH for *O. oeni.*

4.3. Presumption of an antisense targeting mae: identification of a new mechanism for regulating the cit locus expression?

The present study has revealed that the transcription start site of *citR* is located in the coding sequence of *mae* (570 bp upstream of the *citR* start codon), suggesting the synthesis of an antisense overlapping 437 bp of the 5′-end *mae* mRNA (Fig. 1). asRNA represents a non-negligible part of the bacterial transcriptome. Indeed, it has been proven that asRNA accounts for 2.9 % of the transcripts detected in *Bacillus subtilis*, for example, but the proportion may be higher in other bacteria (Georg and Hess, 2011; Rasmussen et al., 2009). These asRNA may be short, from only 100 to 300 nucleotides or even shorter (*e.g.*, GadY in *Escherichia coli* (Opdyke et al., 2004)) or much longer, up to more than 7000 nucleotides, covering several genes of an operon (*e.g.*, asRNA targeting the operon responsible for flagellum biosynthesis in *Listeria monocytogenes* (Toledo-Arana et al., 2009)) (Georg and Hess, 2011; Sesto et al., 2013). The synthesis of these asRNA regulates gene expression *via* various mechanisms, acting at the transcriptional, post-transcriptional or translational levels (Georg and Hess, 2011; Lasa et al., 2012; Sesto et al., 2013; Thomason and Storz, 2010). In our case, the conformation recalls the excludon concept described by Sesto and collaborators (Sesto et al., 2013), in which a transcript composed of an mRNA (here *citR* mRNA) and an asRNA (here the 437 bp asRNA annealing *mae*) results in the repression of the expression of overlapping genes organized in an operon and positioned in divergent directions (in this case *mae* expression).

Monitoring of *cit* locus expression in PSU-1 revealed a significant increase in the number of *citR* transcripts after 24 h of growth, correlated with a huge increase in *maeP* transcription (Fig. 6A). The same results could be obtained for ATCC BAA-1163 and NB_1858 (Fig. S5). Unexpectedly, Cts values obtained after 8 h of growth for NB_1858 were quite different among biological replicates (Fig. S5). Consequently, Cts values for *maeP* varied considerably, while Cts for *mae* and *citE* remained fairly stable. Indeed, it seems that the greater the quantity of *citR* transcripts, the greater the number of *maeP* transcripts. A growth time of 24 h is the moment when citrate consumption by the bacterium is the fastest except for ATCC BAA-1163 (Fig. 2B). This strain consumed citrate in only 16 h because it expressed the *cit* locus more highly throughout growth than the other strains.

Therefore, it might be the case that in *O. oeni*, citrate consumption is controlled by the citrate uptake *via maeP* expression, and that *citR* mRNA could positively control *maeP* transcription. However, only one promoter region was identified for the transcription of the *mae-maeP-citCDEFXG* cluster, which raises the question of how *citR* mRNA could specifically impact *maeP* transcription. Regarding *L. lactis* bv. diacetylactis, it has been reported that CitR could act at the post-transcriptional level on *citP* expression, the ortholog of *maeP* (de Felipe et al., 1994). In our case, a putative cleavage site was detected by the 5′-RACE technique (GGGC/AGG) and is positioned on an air-pin structure (Fig. 1B). It appeared that the two primers used for qPCR analysis annealing *maeP* were positioned on either side of the putative cleavage site (Fig. 6D). A second qPCR analysis was undertaken using two different pairs of primers annealing to the 3′-end of *maeP* (Fig. 6B and C). This experiment clearly highlighted a difference in the number of transcripts detected between the 5′ and the 3′-end of the gene after 8 h of growth, while no significant difference could be demonstrated after 24 h. These results lend further support for the existence of a post-transcriptional regulation mechanism that may cleave the *maeP* transcript between the two primers annealing to the 5′-end (named maePqP-F and maePpP-R, see Table S1) used for qPCR analysis. This regulation mechanism would seem to repress *maeP* expression and possibly the translation of the downstream transcripts encoding for the citrate lyase. This would explain why PSU-1 and NB_1858 did not consume citrate between 0 and 24 h of growth. After 24 h, the level of *citR* transcription has increased along with *mae* asRNA, which seems to reduce the cleavage of the *maeP* transcript.

The complementation of ATCC BAA-1163 with a functional *citR* gene confirmed that the positive effect seen on the *maeP* transcript comes from *mae* asRNA. Indeed, the DNA fragment cloned in the pSIPSYN vector comprises the coding sequence of *citR* as well as the intergenic region between *citR* and *mae* (excluding the *mae* coding sequence targeted by the asRNA) and its expression is under the control of a constitutive promoter. *citR* is in fact much more strongly expressed in OocitR-PSU and OocitR-NB than in Ooc (Fig. 3C & D) but does not result in an increase in the *maeP* transcript, but rather the contrary (Fig. 3C and D). This confirms that CitR is not at the origin of the *maeP* transcript stabilization, focusing attention to the *mae* asRNA. Many studies have described the direct effect of asRNAs on the expression of their overlapping genes that can either be positive or negative (Thomason and Storz, 2010; Georg and Hess, 2011; Lasa et al., 2012; Sesto et al., 2013; Toledo-Arana and Lasa, 2020). Here, an indirect effect of *mae* asRNA on *maeP* transcript is highlighted. We suggest that the interaction between the *mae* asRNA and its target could modify the secondary structure of the polycistronic mRNA and thus prevent recognition of the cleavage site on *maeP* by the endonuclease (Fig. 5). This kind of regulatory mechanism has already been described in other Gram-positive bacteria such as *Staphylococcus aureus* (Durand et al., 2015). Indeed, it possesses a regulatory RNAIII which can form a complex with several mRNA and consequently repress or induce their expression. For example, RNAIII binds the 5'UTR of *hla* mRNA, encoding α-hemolysin, to impair the formation of a secondary structure which normally traps the Shine-Dalgarno sequence of the *hla* gene (Morfeldt et al., 1995). We can also quote the RoxS sRNA from *B. subtilis*, which is able to bind the extreme 5′ end of the *yflS* mRNA, encoding a malate transporter. Fixation of the sRNA stabilize *yflS* mRNA by blocking its degradation by RNase J1 (Durand et al., 2017). A similar mechanism may be implied in the regulation of the expression of the citrate transporter *maeP* in *O. oeni*. To verify this hypothesis, the *mae* asRNA could be integrated in the pSIPSYN plasmid to be constitutively expressed in *O. oeni*. Then, a similar transcriptional analysis by qPCR could comfort the impact of this asRNA on *maeP* transcript level.

Concerning the increase in the *citR* transcript (therefore asRNA) at 24 h of growth which occurred in the three strains studied, it is probably modulated by the bacterial environment. Interestingly, all these strains finished consuming malate at this specific time (Fig. S4). This organic acid is the favored substrate of *O. oeni* because it is the first to be consumed by the bacteria, whatever the growth conditions (Contreras et al., 2018; Saguir and Manca De Nadra, 2002; Viljakainen and Laakso, 2000). Malate may act as a sensor on *cit* locus expression and the absence of malate could activate the transcription of *citR* (and thus *mae* asRNA), increasing *maeP* transcription and initiating citrate consumption in the bacterium.

5. Conclusion

To our knowledge, this is the first time that the expression of the *cit* locus in *O. oeni* has been studied. Transcription start sites are here identified, one before *mae* enabling the transcription of the entire *cit* operon (*mae-maeP-citCDEFXG*) and the second one in the divergent direction enabling *citR* transcription. In addition, we have demonstrated that the *citR* mRNA that overlaps the *cit* operon transcript constitutes an asRNA targeting *mae*. This asRNA, whose expression is upregulated after 24 h of growth, could indirectly prevent the cleavage of *maeP* mRNA by endonuclease, resulting in the initiation of citrate consumption in

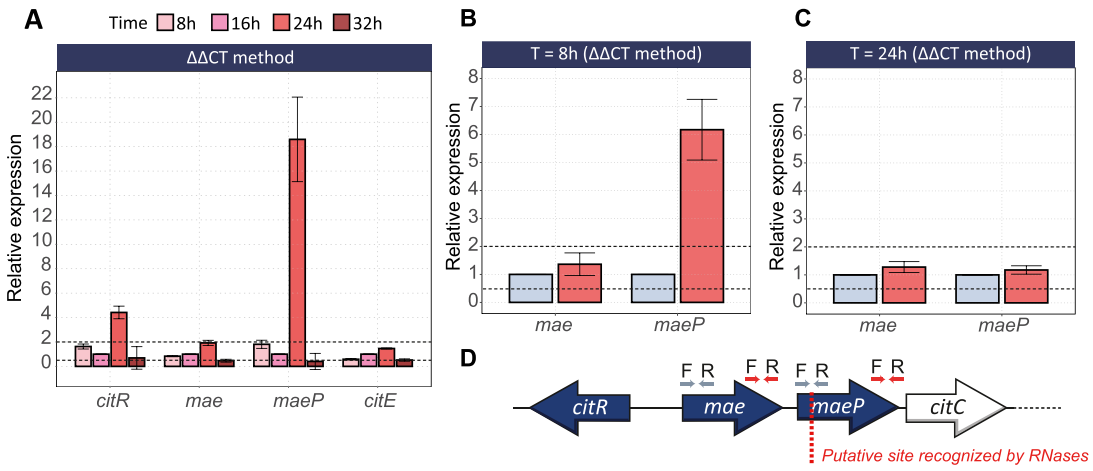


Fig. 6. Expression of citrate genes in PSU-1. Total RNA was extracted from PSU-1 culture grown in GEMO medium at pH 4.8 after 8-, 16-, 24- and 32-h of growth. (A) Expression level of the citrate genes related to time = 16-h of growth, used as a reference (housekeeping genes are *ldh* and *gyrA*, ΔΔCt method). (B and C) Expression level of *mae* and *maeP* depending on the position of the primers on the gene (B = after 8 h of growth and C = after 24 h of growth). Ct of *mae* and *maeP* were first related to the Ct of housekeeping genes *ldh* and *gyrA*, then ΔCt of *mae* and *maeP* 3′-ends (red bars) were referred to ΔCt of *mae* and *maeP* 5′-ends (grey bars). The position of the primers on the *cit* locus is indicated (D). The results were obtained from biological triplicates run twice on the same plate (technical duplicates). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

O. oeni. Initiation of citrate consumption *via* the regulation of the permease gene has also been suggested in studies of other LAB (Martín et al., 2000, 2004). The activation of the *mae* asRNA transcription may be modulated by the environment and malate could be the molecule controlling this expression, but this lead must be investigated further.

Furthermore, monitoring of *cit* locus expression in three different *O. oeni* strains here revealed that CitR, whose role was unknown until now, must be a transcriptional repressor of *mae-maeP-citCDEFXG* expression. This hypothesis was reinforced by a genetic complementation performed on *O. oeni* ATCC BAA-1163 with a functional *citR* gene. The present work represents the first successful genetic complementation performed on *O. oeni* to demonstrate *in vivo* gene functionality, thus providing a new method for modulating gene expression in this hard-to-handle bacterium, in which only the RNA silencing technique has previously been successfully employed (Darsonval et al., 2015, 2018). Its expression in the complemented strains triggered the downregulation of citrate genes, which resulted in the slowdown of citrate metabolism. This slowdown increased the acid tolerance of the bacterium, providing new evidence for the involvement of citrate metabolism in the acid stress response of *O. oeni*. Therefore, this work highlighted that the regulation of the *cit* locus expression must have a significant impact on the ability of *O. oeni* to conduct MLF in very acid wines. Nevertheless, the molecular mechanism of the action of CitR still needs to be identified.

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CRediT authorship contribution statement

Camille Eicher: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Conceptualization. **Amaury Aumeunier:** Writing – review & editing, Investigation. **Oriane Theveny:** Writing – review & editing, Investigation. **Frédérique Julliat:** Writing – review & editing, Methodology, Investigation. **Tiffany Bellanger:** Writing – review & editing, Investigation. **Joana Coulon:** Writing – review & editing, Resources, Funding acquisition. **Marion Favier:** Writing – review & editing, Resources, Funding acquisition. **Hervé Alexandre:** Writing – review & editing, Supervision. **Cristina Reguant:** Writing – review & editing, Supervision. **Cosette Grandvalet:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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