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FEMS Microbiology Letters 126 (1995) 209–214

FEMS MICROBIOLOGY LETTERS

MiniReview

# Microbiology of the malolactic fermentation: Molecular aspects

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Received 10 October 1994; revised 9 January 1995; accepted 10 January 1995

**Abstract**

Malolactic fermentation conducted by lactic acid bacteria follows alcoholic fermentation during winemaking, and several positive effects make it indispensable for most wines. Research has focused on the growth and physiology of lactic acid bacteria in wine; resulting in the design of malolactic starter cultures. Future work on these starters will concentrate on aromatic changes as additional criteria for strain selection. Although the main features of the malolactic enzyme and its gene are known, the detailed mechanism of the malolactic reaction remains unclear. Cloning and expression of this activity in enological strains of *Saccharomyces cerevisiae* might be one of the next most important advances in the control of malic acid degradation in wine.

*Keywords:* Malolactic fermentation; Lactic acid bacteria; Wine; Fermentation

## 1. Introduction

Winemaking normally comprises two main fermentation processes: alcoholic fermentation conducted by yeasts and malolactic fermentation (MLF) which is performed by various lactic acid bacteria (LAB). The second is essential for nearly all red wines and some white, especially those which undergo barrel or bottle-ageing. MLF improves the quality and stability of these wines. Both yeasts and LAB are common microorganisms in grape must before fermentation, and in wine throughout the vinification process. Normally, LAB cannot grow at the same time as yeasts, but multiply during the final stages of the alcoholic fermentation or just after it. Therefore, the MLF normally follows, within a few days, the alcoholic fermentation if the general conditions are favourable [1].

The main effect of the MLF is a decrease in total acidity resulting from the decarboxylation of L-malic acid to L-lactic acid. MLF induces a dramatic change in the organoleptic quality of wines, since, besides deacidification, the specific taste of malic acid disappears. Moreover, numerous other substrates are metabolized. Sugars are catabolized to produce mainly lactic and acetic acids, and citric acid is transformed into acetic acid and carbonyl compounds, notably the butter-flavoured diacetyl. Wine taste and colour are also modified due to the metabolic activity of bacteria on phenolic compounds (tanins, anthocyans), which are basic components of wines.

Besides the improvement in organoleptic quality, MLF also plays a part in microbial stabilization. Wine spoilage by other bacteria is less frequent when LAB have already developed. This may be explained not only by the deprivation of nutrients, but also probably by the synthesis of antibacterial compounds [2,3].

MLF was recognized as an indispensable step in winemaking more than 40 years ago but, it took


*SSDI* 0378-1097(95)00020-8

more than 20 years to convince winemakers of this. Intensive studies in wine microbiology have now provided knowledge of the main physical and chemical factors affecting the growth of yeasts and bacteria. However, many aspects still remain unclear and practical difficulties are often encountered. For this reason, basic research on LAB and especially on the malolactic enzyme continues.

## 2. Lactic acid bacteria of wine: role in vinification

### 2.1. Bacterial microflora of grape must and wine

When the fermentation tanks are filled after crushing, the grape juice contains yeasts, lactic, and acetic bacteria. Viable populations of $10^2$ to $10^4$ cells/ml of bacteria occur, varying mainly according to the conditions during the final days of ripening and harvest. Addition of sulfite to the grape must is the first step in vinification; it lowers the bacterial population. Yeasts, which are less sensitive to the sulfite, grow rapidly initiating the alcoholic fermentation. During this phase the conditions are very unfavourable for bacterial growth. After a short period of multiplication, interactions between yeasts and bacteria result in a decrease in the bacterial population [4]. At the end of the alcoholic fermentation the numbers are usually about $10^2$–$10^3$ LAB cells/ml.

During alcoholic fermentation the bacterial population is also submitted to a natural selective process. While up to eight different species of LAB have been identified in grape must, generally only one species, *Leuconostoc œnos*, can be isolated at the end of the alcoholic fermentation [5]. Pediococci and lactobacilli might also be involved in MLF, although they generally produce off-flavors. More importantly, these strains may be so resistant to the harsh conditions of wine that they may survive even during storage, which can lead to wine spoilage.

### 2.2. Factors affecting bacterial growth:

Malic acid transformation actually begins when the bacterial population reaches about $10^6$ cells/ml. Therefore, from the end of the alcoholic fermentation, when the level is $10^2$–$10^3$ cells/ml to the beginning of the MLF, a multiplication phase occurs, the duration of which is directly dependent on pH, temperature and ethanol content as the main factors. Growth is easier at relatively high pH (pH $>$ 3.5), with an ethanol content less than 13% and at 19–20°C, and it is nearly impossible at pH $<$ 3.0, ethanol $>$ 14% and below 17°C. However, these figures are not absolute since all these factors and other inhibiting or activating components of wine interact to determine the 'malofermentability' of wine [1]. Normally, when malic acid transformation has begun, it continues to completion. The necessary time varies from 5 days to 2 or 3 weeks according to the physico-chemical conditions of the medium and the quantity of acid to transform.

After a normal start, MLF might suddenly stop, then start again after several days. This unusual pattern is attributed to infection of the viable bacterial population by phages [6]. MLF finishes once the initial bacterial population is replaced by another, which might be mainly composed of pediococci. Like other bacteria, *L. œnos* strains may be lysogenic, and sensitive or resistant to phage attack. Our recent results have shown that up to 90% of *L. œnos* strains isolated from wine are lysogens. Spontaneous induction of phage from these lysogenic strains is variable according to the strain. Although about $10^2$–$10^3$ phages/ml are commonly encountered during MLF (Poblet and Lonvaud-Funel, unpublished results), these levels are insufficient to completely destroy the bacteria. Indeed, erradication by phage is very unlikely in spontaneous MLF, since different strains of *L. œnos* and different phages are present and even if one strain is totally eliminated by a phage attack, the others can multiply and continue MLF. However, MLF arrest due to phages cannot be excluded.

### 2.3. Use of malolactic starter cultures:

After the alcoholic fermentation and as long as MLF has not begun, wine cannot be treated by sulfiting which would inhibit LAB growth. During this interim period the wine is exposed to chemical oxidation or development of spoilage microorganisms such as acetic acid bacteria and yeasts. Therefore the delay between the two fermentations must be as short as possible. To save time and to prevent

spoilage, winemakers use malolactic starter cultures. Several lyophilized industrial starters are now commercially available, the most efficient being produced from *L. œnos* strains originally isolated from wine. Until recently, these starter cultures needed a reactivation step before inoculation into the wine but a 'ready-to-use' lyophilized preparation is now available. The preparation is suspended directly in wine and, unlike the previous industrial products, the bacterial population retains its viability during the first days and finally grows, inducing malic acid degradation within 5–7 days. *L. œnos* starter cultures must be inoculated after completion of the alcoholic fermentation to avoid sugar metabolism which would increase the volatile acidity of the wine. Sensory evaluations of wines inoculated with different strains of *L. œnos* have shown some specific strain influence, although the differences are neither particularly significant nor reproducible.

## 3. Biochemistry of malolactic fermentation

### *3.1. Mechanism of malic acid transformation*

The term 'malolactic fermentation' was given to this step of winemaking by analogy to alcoholic fermentation long before the real mechanism was discovered. From a biochemical point of view, the conversion of malic acid to lactic acid is not a fermentative step, since it only consists in a decarboxylation :

However, at the same time, the residual sugars which are not fermented by the yeasts, are fermented by bacteria. Glucose, fructose, xylose and arabinose are metabolized by *L. œnos* by the heterofermentative pathway to produce D-lactic acid, acetic acid, ethanol and $CO_2$ as main products. The assumption that LAB use a novel mechanism for malic acid degradation was suggested subsequent to the observations of Peynaud et al. that L-lactate was the only product of the reaction with $CO_2$ [7]. This hypothesis was based on the stoechiometry and stereochemistry of the reaction. One molecule of the L-isomer of malic acid, the natural form of the acid in wine, was observed to produce one molecule of L-lactic acid and one molecule of $CO_2$. Yet heterofermentative cocci such as *L. œnos* produce only the D-isomer of lactic acid from sugars, so these strains contain only the D-lactate dehydrogenase which catalyses the reduction of pyruvate to D-lactic acid. Therefore, malic acid conversion does not involve either the well known malate dehydrogenase or the malic enzyme which produce pyruvic acid from L-malic acid. During MLF, a novel enzyme catalyses the decarboxylation of L-malic acid without production of pyruvic acid as an intermediary free product. This enzyme has been termed malolactic enzyme (MLE) [8]. It was first purified from LAB species usually present in grape must or wine [9–11,11,13], then also described in other kinds of LAB from various origins [14,15].

### *3.2. Energetics of MLF:*

When research on MLF started, the most common result described by several authors was that bacteria grew more easily in media with added L-malic acid. Nevertheless, the malolactic reaction by itself does not provide energy to the cell. The most usual comment was that in an acidic medium like wine, the decarboxylation of L-malic acid induced an increase in pH which was beneficial to bacterial growth. However, recent results have demonstrated that in an acidic medium, the malolactic reaction induces ATP production via a chemiosmotic mechanism. The transport of L-malic acid into the cell, its decarboxylation and efflux as L-lactic acid and the concomittant efflux of a proton create an energetic proton motive force. Thus, the transfer of L-malate and L-lactate through the membrane as the result of MLF generates energy, which explains the improvement of bacterial growth in such a medium.

## 4. The malolactic enzyme

### *4.1. Main features:*

The malolactic enzyme (MLE) has been purified from various LAB species isolated from grapes and wines or from collection strains including *Lactobacillus* sp. [9,10,13,14] and *Leuconostoc* sp. [11,12] as well as *Lactococcus lactis* (Lonvaud-Funel, unpublished results). The enzyme is inducible by L-malate. Its purification normally involves a fractionated precipitation with ammonium sulfate, then exclusion filtration, ion exchange, adsorption and affin-

ity chromatography, chromatofocusing or isoelectric focusing according to the authors.

Pure MLE catalyses the transformation of one molecule of L-malic acid into one molecule of L-lactic acid. The same pure fractions are unable to catalyse the reduction of pyruvic acid to L- or D-lactic acid. The main traits of the enzyme, kinetic parameters, effectors and properties have been described [11,12,16].

### *4.2. Cloning and sequencing of the mleS gene*

The structural gene for MLE from *L. lactis* IL 1441, has been sequenced since only this strain has been studied genetically [15]. The two different strategies used for cloning this gene were based on the primary N-terminal amino-acid sequence of the purified MLE (Lonvaud-Funel, unpublished results).

Ansanay et al. [17] cloned the *mleS* gene by screening a DNA genomic library of *L. lactis* in lgt 11 using a polyclonal serum against MLE. Positive clones were then hybridized and selected using a degenerate probe deduced from the N-terminal amino-acid sequence. A 2.7 kb insert of a clone containing the coding region was sequenced. Denayrolles et al. [18] synthesized by PCR a specific probe EML60 corresponding to the N- terminal sequence : the primers were deduced from the five amino-acids at each end of the known 20-amino-acid sequence. EML60 was used as a probe in Southern hybridization of *Eco*RI, *Eco*RV and *Hin*d III digests of the genomic DNA. Several patterns were obtained showing that only one sequence on the chromosome hybridized with EML60. Finally, from three partial genomic libraries, two fragments were isolated which included the *mleS* gene and the flanking regions. The complete sequence was determined on both strands for 1927 bp. The authors of the two papers describe the same open reading frame (ORF) of 1620 nucleotides encoding a putative protein of 540 amino-acids with a theoretical molecular weight of 59 kDa, identical to that of the subunit determined by gel electrophoresis. The calculated pI is 4.46 which is in agreement with the measurements of several authors. A potential ribosome-binding site at position -12 was identified. No terminator was identified for the *mleS* gene; it is followed by another ORF which has no promoter. Therefore it is assumed that the malolactic locus comprises an operon structure [17,18].

### *4.3. Analysis of the deduced amino-acid sequence: search for homologies with malic enzymes*

The deduced amino-acid sequence of *mleS* is highly homologous with malic enzymes (ME) of several organisms. The strongest homology was found with the $NAD^+$ dependent ME of *E. coli*; throughout a sequence of 488 amino acids, the similitarity was 39.7%. A region of 8 amino acids which is found in MLE and is known as the ME signature, is strictly conserved between the eight sequences compared. Two regions match the consensus sequence for ADP binding ß $\alpha$ ß-fold sites [18]. These are probably involved in binding the ADP moeity of the cofactor NAD. Finally, another consensus sequence could be the site for substrate binding when compared to ME [18]. However, in this region MLE differs from most of the MEs especially by substitution of an isoleucine residue by a cysteine, which is reported as being important for the binding of L-malate and $Mn^{2+}$. This replacement is also reported in the case of the ME of *Schizosaccharomyces pombe* [19]. When this cysteine residue is alkylated by bromopyruvate, the ME of duck liver loses its malic activity and increases its secondary L-LDH activity [20]. This is particularly striking since the difference between ME and MLE is the additional L-LDH activity of the latter. It has long been thought that MLE acts as a multienzymatic complex of ME plus L-LDH; this hypothesis is now refuted since only one structural gene encodes MLE.

## 5. Future prospects for controlling MLF

### *5.1. Use of malolactic starter cultures*

Several winemaking operations are used to obtain better control of MLF. Temperature is of course the easiest parameter to control. In many cases, keeping the temperature around 20°C promotes bacterial growth. Also, the technique of chemically deacidifying wine, thus encouraging LAB growth, is currently used to stimulate 'difficult' wines. However, winemakers can now use industrial malolactic starter cultures which are all prepared with selected *L. œnos* strains. At the moment, only one ready-to-use strain is commercially available, and this constitutes a real

advance in wine technology. However, winemakers now need other strains. Although malic acid degradation seems now to be under control, the organoleptic effects of MLF might also be manipulated by judicious choice of the strain.

### 5.2. Malolactic reactors:

Immobilized LAB or MLE could be a solution for MLF. Divies and Siess [21] first described the conversion of L-malic acid by entrapped *Lactobacillus casei* in a synthetic medium. The reactor was continuously supplied with the nutrient medium for survival and activity of the bacteria. Many other trials have been reported: most show that MLF can be induced in synthetic media without any problem. However, the reactor needs regeneration and the initial problem of bacterial growth in wine is again posed. Probably the most significant trials from the enological point of view were those reported by Cuenat and Villetaz [22]. Infection of the reactor by spoilage bacteria or by phages, the loss of malolactic activity, and undesirable changes in organoleptic quality are the major objections to this technology.

Entrapped MLE in carraghenan or alginate has also been used. Obviously these reactors can only work in synthetic media since the enzyme needs cofactors and a pH value of around 6.0 for its activity. Nevertheless, real progress has been made by Festaz-Furet [23] who took into account the constraints of the medium for the activity of the enzyme. The enzyme was blocked in the internal compartment of a hollow fiber reactor and NAD was immobilized on the membrane at the suitable pH. When wine was circulated in the external compartment, malic acid diffused inside the fiber where the reaction took place and lactic acid diffused out. This is the first description of a MLE reactor which deals with the most important parameters needed for enzyme activity. However, more research is necessary to improve these preliminary results.

### 5.3. Cloning of mleS in Saccharomyces cerevisae

While growth of indigenous LAB or malolactic starters in wine is hazardous, yeasts develop very easily in grape must. For this reason, inoculation of must by yeast carrying malolactic activity was suggested as soon as the genetic improvement of microorganisms became feasible. The modified yeast could perform the alcoholic fermentation of sugars and the malic acid degradation simultaneously. Therefore, the wine would be ready for stabilization and ageing as soon as alcoholic fermentation was finished.

The first attempt was the cloning of the gene for malolactic activity of *Lactobacillus delbrueckii* into *S. cerevisiae* by Williams et al. [24]. Only 1% of L-malate was converted into L-lactate. Cloning in *E. coli* of the same DNA fragment led to 10% conversion in anaerobiosis. Lautensach and Subden [25] prepared a genomic library of *L. œnos* in a strain of *E. coli* which was unable to use L-malate. The selected clones were unstable and it could not be proved that L-malate was converted into L-lactate, a step characterizing malolactic activity.

A completely different strategy was chosen some ten years after these initial studies. After the malolactic gene was isolated, it was cloned and expressed in *E. coli* [17,18]. It was found that recombinant clones could produce L-lactate. The gene was also cloned by both teams in *S. cerevisiae*. A transformant grown in a medium containing 10 g/l L-malate produced 0.52 g/l L-lactate while the control strain produced only traces [17]. Crude extracts of several transformed *S. cerevisiae* lines converted, almost stocheiometrically, within 4 hours from 13 to 29 $\mu$mol of L-malate into the corresponding 9 to 18 $\mu$mol of L-lactate. At the same time, the control strain did not degrade L-malic acid [18]. One of these strains inoculated in grape must conducted alcoholic fermentation at the same rate as the control with the same yield (sugar/ethanol). However of 4.5 g/l L-malic acid, only 7.5% was transformed by the MLE [18].

## 6. Conclusion

Even if MLF is the second step in winemaking, it is far from being of secondary importance. It improves wine quality and stability. Usually the spontaneous development of the indigenous bacteria achieves total malic acid degradation. At present, the process can also be started by inoculation of selected strains. So far, the ability of starter cultures to sur-

vive in wine has been the only test of their efficiency. However, organoleptic changes seem to be strain-specific. More research is needed on this new criterion, especially concerning the main aromatic compounds of bacterial metabolism.

The MLE, which is the most important enzyme of LAB from the enological point of view, is an intriguing enzyme and its mechanism of action is not yet fully understood. The cloning of the gene in *S. cerevisiae* and its effective expression are among the most exciting challenges today in enology. During alcoholic fermentation such transformed yeasts could degrade malic acid, and their use in winemaking would be most beneficial when only deacidification is required, since wine aroma would in no way be jeopardised.

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