Effect of alpha-acetolactate inactivation on alpha-acetolactate and diacetyl production by Lactococcus lactis subsp. lactis biovar diacetylactis

F. Aymes, Christophe C. Monnet, Georges Corrieu

To cite this version:
F. Aymes, Christophe C. Monnet, Georges Corrieu. Effect of alpha-acetolactate inactivation on alpha-acetolactate and diacetyl production by Lactococcus lactis subsp. lactis biovar diacetylactis. Journal of Bioscience and Bioengineering, 1999, 87 (1), pp.87-92. hal-02696631

HAL Id: hal-02696631
https://hal.inrae.fr/hal-02696631
Submitted on 1 Jun 2020
Effect of $\alpha$-acetolactate decarboxylase inactivation on $\alpha$-acetolactate and diacetyl production by Lactococcus lactis subsp. lactis biovar diacetylactis.

Running title: $\alpha$-Acetolactate production by Lactococcus lactis.

Frédéric AYMES, Christophe MONNET* and Georges CORRIEU

Laboratoire de Génie et Microbiologie des Procédés Alimentaires
Institut National de la Recherche Agronomique
78850 Thiverval-Grignon, France

*Corresponding author.
Phone: (33) 1 30 81 54 91. Fax: (33) 1 30 81 55 97.
E-mail: monnet@cardere.grignon.inra.fr

**Key words:** Lactococcus, diacetyl, $\alpha$-acetolactate, $\alpha$-acetolactate decarboxylase.
Strains of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* deficient in \( \alpha \)-acetolactate decarboxylase produce \( \alpha \)-acetolactate. This unstable compound is a precursor of acetoin and an aromatic compound, diacetyl. Following random mutagenesis of strain CNRZ 483, \( \alpha \)-acetolactate decarboxylase-negative mutant 483 M1 was selected. When grown in milk, its growth and acidification characteristics were similar to those of the parental strain. In anaerobic conditions, the parental strain produced 2.10 mM acetoin and less than 0.05 mM diacetyl. The mutant accumulated up to 2.11 mM \( \alpha \)-acetolactate, which spontaneously degraded to acetoin and diacetyl. After 24 hours of culture, the \( \alpha \)-acetolactate concentration was only 0.49 mM and the acetoin and diacetyl concentrations reached 1.50 mM and 0.26 mM, respectively. Diacetyl production by both strains increased in aerobic conditions, as well as when citrate was added. In contrast to cultures of the parental strain, however, diacetyl and acetoin concentrations in mutant cultures continued to increase without reaching a plateau. The results also showed that diacetyl production by wild type *L. lactis* subsp. *lactis* biovar *diacetylactis* strains cannot be explained uniquely by the spontaneous decarboxylation of the \( \alpha \)-acetolactate produced in the culture medium.
INTRODUCTION

Diacetyl is a major flavour component of several fermented dairy products including cultured cream, cultured butter, buttermilk, and fresh cheeses. It is an end-product of citrate metabolism by certain lactic acid bacteria such as Lactococcus lactis subsp. lactis biovar diacetylactis and Leuconostoc sp. Diacetyl arises from the spontaneous oxidative decarboxylation of α-acetolactate (7, 10, 24), which can also be transformed to acetoin by α-acetolactate decarboxylase or by spontaneous non-oxidative decarboxylation (Fig. 1).

Since lactic acid bacteria produce relatively small quantities of diacetyl (generally less than 0.05 mM), considerable work has been devoted to increasing the production of this compound. Factors such as pH (20, 22), citrate concentration (4, 15) and oxygen (2, 3) have been studied for their effects on diacetyl production. Regardless of culture conditions, however, acetoin is consistently produced in higher concentrations than diacetyl. This results from α-acetolactate decarboxylase activity, which transforms α-acetolactate to acetoin.

When α-acetolactate decarboxylase-deficient strains are used, α-acetolactate accumulates in the culture medium, resulting in higher diacetyl production, especially in aerobic conditions (10, 17). Starter culture 4/25, known for its ability to produce high concentrations of diacetyl in the butter-making process (25), contains a naturally occurring α-acetolactate decarboxylase-negative L. lactis subsp. lactis biovar diacetylactis (10, 12, 24).

These deficient strains can be obtained by disruption of the aldB gene (8) or after random mutagenesis (18). Goupil et al. (9) have devised a method for selecting spontaneous α-acetolactate decarboxylase-negative mutants, applicable to L. lactis strains prototrophic for branched-chain amino acids. The application of these techniques to strains having interesting technological properties such as high acidification activity or bacteriophage resistance, could
lead to the improvement of some dairy processes. It is thus likely that the industrial use of 
\( \alpha \)-acetolactate decarboxylase-negative mutants of *L. lactis* subsp. *lactis* biovar *diacetylactis* will expand in the near future. For the moment, however, relatively little data are available concerning the behavior of these mutants in milk cultures. The purpose of this study was to
determine the effect of \( \alpha \)-acetolactate decarboxylase inactivation, obtained by random mutagenesis, on the growth of *L. lactis* subsp. *lactis* biovar *diacetylactis* in milk. This effect was studied in anaerobic cultures and in conditions more favorable to diacetyl production, e.g.
in the presence of oxygen and by supplementing the medium with citrate.
MATERIALS AND METHODS

**Strains**  
*L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 was obtained from the collection of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France). 

α-acetolactate decarboxylase-negative mutant 483 M1 was selected after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (18).

**Culture conditions**  
Ten liters of UHT (ultra high temperature) sterilized skim milk (Candia, Lyon, France) were inoculated at 3% (v/v) with a freshly prepared culture grown in the same medium for 15 h at 30°C. The 15 liter reactor (Biolaffite, Saint-Germain-en-Laye, France) was maintained at 30°C and stirred at 400 rpm. The oxygen concentration and pH were measured on-line with appropriate probes (Mettler Toledo, Urdorf, Switzerland) and monitored with a data acquisition system. Zero per cent oxygen was maintained with nitrogen, and oxygen intake was ensured by pressurizing the headspace above the culture medium at 2 atmospheres (absolute pressure) of oxygen (3). Compared to the control of oxygen concentration with a permanent oxygen flow through the medium, this procedure avoided foam formation in the reactor. Under a pressure of 2 atmospheres, the dissolved oxygen concentration remained between 90% and 100% of medium saturation. The endogenous citrate concentration of the milk used was 9 mM. A higher citrate concentration (23 mM) was obtained by adding 14 mM trisodium citrate dihydrate (Prolabo, Paris, France).

**Analyses**  
Growth was monitored at 405 nm with a spectrophotometer (UV-2100, Shimadzu, Kyoto, Japan) after clarification of the milk sample with 0.2% EDTA, pH 12.5 (1/10, v/v) (14).
Diacetyl and \( \alpha \)-acetolactate were determined as described by Mohr et al. (16). The sum of diacetyl plus acetoin was determined by the method of Westerfeld (26).

Cell-free supernatant fluids, obtained by precipitating a 1.8 ml sample with 120 \( \mu \)l of trichloroacetic acid (400 g/l) and centrifuging for 20 min at 16,000 \( \times \) g, were used to assay citrate, lactate, acetate, formate, ethanol and 2,3-butanediol by HPLC (2).

**Determination of kinetic parameters** Maximum growth rates (h\(^{-1}\)) were determined by the slope of the linear portion of the plot of the natural logarithm of absorbance values vs. time.

Maximum acidification rates (pH unit.h\(^{-1}\)) were determined using a Weibull function as previously described (2).

The rates of \( \alpha \)-acetolactate decarboxylation (h\(^{-1}\)) were determined by the slope of the plot of the natural logarithm of \( \alpha \)-acetolactate concentrations vs. time, after citrate depletion and until the end of the fermentation.

**Enzyme assays** Lactate dehydrogenase, NADH oxidase, acetoin reductase, diacetyl reductase, \( \alpha \)-acetolactate synthase and \( \alpha \)-acetolactate decarboxylase activities were measured in cell-free extracts according to the procedures described by Boumerdassi et al. (5).
RESULTS

Selection of an $\alpha$-acetolactate decarboxylase-negative mutant  The method used to obtain $\alpha$-acetolactate decarboxylase-negative mutants, described by Monnet et al. (18), is based on treatment with the mutagen N-methyl-N$'$-nitro-N-nitrosoguanidine (NTG), followed by screening on Petri dishes. The survival rate of strain CNRZ 483 was close to 10% after treatment with NTG at 300 $\mu$g.ml$^{-1}$. This concentration was used to create mutants producing $\alpha$-acetolactate. After screening 15,000 colonies, one positive clone was isolated (483 M1). Mutant 483 M1 has no detectable $\alpha$-acetolactate decarboxylase activity (< 0.01 U.mg$^{-1}$ of protein), while the activity of the parental strain CNRZ 483 was 0.64 U.mg$^{-1}$ of protein. None of the other five enzymatic activities measured ($\alpha$-acetolactate synthase, lactate dehydrogenase, NADH oxidase, acetoin reductase and diacetyl reductase) was significantly modified by treatment with the mutagen (results not shown).

Stability of the $\alpha$-acetolactate decarboxylase negative mutant  Mutant 483 M1 was subjected to subcultures in M17 broth (23) or UHT sterilized skim milk. Each culture (10 ml) was inoculated at 1% and incubated under static conditions for one day at 30°C. After 20 successive subcultures, which corresponded approximately to 130 generations, the cultures were diluted and inoculated on agar plates of the medium for screening $\alpha$-acetolactate producing colonies (18). More than 500 colonies were examined for each type of culture. No reversion of the $\alpha$-acetolactate production phenotype could be detected in these conditions.

Effect of culture conditions on growth, acidification and citrate utilization  Three culture conditions were tested: anaerobiosis, aerobiosis, aerobic cultures supplemented with
14 mM citrate. In these conditions, the curves of absorbance (A), pH and the citrate content of
the two strains had the same shape (Fig. 2). In cultures not supplemented with citrate, the final
A was close to 5.5 in both the absence and presence of oxygen (Table 1). The maximal growth
rate of both strains, however, was higher in the absence of oxygen. The addition of citrate did
not significantly change the growth rate, but increased the final A of cultures. The presence of
oxygen, and to a lesser extent the addition of citrate, reduced the maximal acidification rate of
the medium and increased the final pH value (24 h of culture). The presence of oxygen and
the addition of 14 mM citrate, delayed citrate exhaustion of the medium.

Effect of culture conditions on the production of lactate and acetate

In anaerobiosis, maximal lactate production by L. lactis subsp. lactis biovar diacetylactis CNRZ
483 and the α-acetolactate decarboxylase-negative mutant 483 M1 were 74.5 and 81.0 mM,
respectively (Fig. 3). The rate of this production was considerably reduced and the quantities
formed were lower in the presence of oxygen. In the presence of oxygen, the addition of
citrate increased the final lactate concentration for both strains.

Acetate is produced in lactococci by the cleavage of citrate by citrate lyase in a
stoichiometric ratio. Acetate production in anaerobiosis was thus close to 10 mM (Fig. 3). Production was stimulated slightly by oxygen in cultures of the parental strain CNRZ 483. In aerobic cultures supplemented with 14 mM citrate, acetate production was close to the initial
citrate concentration. The production of formate or ethanol could not be detected in these
cultures.

Effect of culture conditions on the production of diacetyl, acetoin and
α-acetolactate

Diacetyl production by the parental strain CNRZ 483 was very low in
anaerobiosis (<0.05 mM) (Fig. 4 a). In the presence of oxygen, diacetyl production started
within several hours and increased to a maximum of 0.70 mM, corresponding to the exhaustion of citrate. In the presence of oxygen, the addition of citrate increased diacetyl production. In aerobiosis, diacetyl production was proportional to citrate consumption (Fig. 5). The slope of the regression line of diacetyl production as a function of citrate consumption was 0.07 in aerobiosis and 0.06 in the aerobic culture supplemented with citrate. Acetoin accumulated within the first few hours of culture (Fig. 4 b), its concentration reaching a maximal value of 2.10 mM in anaerobiosis. Higher values were obtained in the presence of oxygen and when citrate was added. As for diacetyl production, the end of the acetoin production corresponded to the exhaustion of citrate in the culture medium. In addition, \( \alpha \)-acetolactate (Fig. 4 c) or 2,3-butanediol production could not be detected in cultures of strain CNRZ 483.

The \( \alpha \)-acetolactate decarboxylase-negative mutant 483 M1 produced \( \alpha \)-acetolactate within the first few hours of culture (Fig. 4 f). The maximal \( \alpha \)-acetolactate concentration was reached when citrate was exhausted; 2.11 mM was produced in anaerobiosis. Higher values were obtained in the presence of oxygen and when citrate was added. The \( \alpha \)-acetolactate concentration then progressively decreased according to a first order chemical reaction. The rate of \( \alpha \)-acetolactate breakdown was 0.095 h\(^{-1}\) in anaerobiosis, 0.056 h\(^{-1}\) in aerobiosis and 0.061 h\(^{-1}\) in the aerobic culture supplemented with citrate. The more rapid breakdown of \( \alpha \)-acetolactate in anaerobiosis was probably due to the lower pH of the medium. The diacetyl and acetoin concentrations increased regularly during the 24 hours of culture (Fig. 4 d and e). Final diacetyl concentrations were 0.26, 0.78 and 1.50 mM in anaerobiosis, in presence of oxygen, and when 14 mM citrate was added in presence of oxygen, respectively. Diacetyl production was much slower than for the parental strain, but continued after citrate was exhausted (Fig. 5). Thus, after 24 hours of culture, the maximal diacetyl concentration had not
yet been reached, since α-acetolactate was not entirely degraded. In the three culture conditions, the amount of α-acetolactate degraded between the time of citrate exhaustion and the end of fermentation (Fig. 4 f) roughly corresponded to the sum of the diacetyl and acetoin produced during the same period (Fig. 4 d and e).

If we suppose that diacetyl was not reduced to acetoin by diacetyl reductase, the diacetyl/(diacetyl plus acetoin) ratio is the proportion of degraded α-acetolactate which underwent oxidative decarboxylation. At the end of culture of the parental strain CNRZ 483, this ratio was lower than 1% in anaerobiosis, and on average 14% in presence of oxygen (Table 2). The ratio for the α-acetolactate decarboxylase-negative mutant 483 M1 was 14.8% in anaerobiosis, and close to 30% in presence of oxygen.

Total production of diacetyl, acetoin and α-acetolactate is proportional to the quantity of pyruvate metabolised via the α-acetolactate synthase pathway. In the three culture conditions tested, the maximal concentrations reached by strain CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1 were similar (Table 2). Nevertheless, total production of diacetyl, acetoin and α-acetolactate increased in the presence of oxygen, as well as when 14 mM citrate was added.
DISCUSSION

In the three culture conditions examined, the growth of mutant 483 M1 was similar to that of the parental strain CNRZ 483. The inactivation of $\alpha$-acetolactate decarboxylase thus did not affect the growth of *L. lactis* subsp. *lactis* biovar *diacetylactis* in milk. Random mutagenesis, however, could create mutations not screened for, and which could limit growth of the strains. This is why it is important to verify that the growth characteristics of the mutants selected after random mutagenesis are similar to those of the parental strains. The $\alpha$-acetolactate decarboxylase-negative mutant 483 M1 and strain CNRZ 483 exhibited no differences in the acidification of milk, citrate consumption or the production of lactate and acetate. The $\alpha$-acetolactate production phenotype of mutant 483 M1 was stable over more than 20 successive subcultures. This stability might be due to the fact that, as observed by comparing strain 483 M1 with strain CNRZ 483, $\alpha$-acetolactate decarboxylase positive revertants probably don’t grow much faster than $\alpha$-acetolactate decarboxylase negative mutants. Possible revertants would thus fail to outgrow rapidly the mutant.

Total production of diacetyl, acetoin and $\alpha$-acetolactate is proportional to the quantity of pyruvate metabolized via the $\alpha$-acetolactate synthase pathway. It was equivalent in the two strains but varied with experimental conditions. The presence of oxygen and the addition of citrate to the medium increased the quantity of pyruvate shunted towards $\alpha$-acetolactate, probably by increasing the intracellular pyruvate concentration and $\alpha$-acetolactate synthase activity (2, 3, 6). The proportions of diacetyl, acetoin and $\alpha$-acetolactate in cultures of the $\alpha$-acetolactate decarboxylase-negative mutant 483M1 were different from those of the parental strain. Mutant 483 M1 produced $\alpha$-acetolactate until exhaustion of citrate. $\alpha$-Acetolactate was then spontaneously degraded to diacetyl and acetoin and there was a shift between citrate...
consumption and the production of diacetyl and acetoin. As a result of this, the maximal diacetyl concentration was not reached after 24 hours of culture. It would be possible to obtain a higher diacetyl concentration in cultures of strain 483 M1 by using a chemical conversion step of \(\alpha\)-acetolactate at the maximum of its production, by heating (11) or acidification of the medium (25). The diacetyl/(diacetyl plus acetoin) ratio reflected the oxidative decarboxylation of \(\alpha\)-acetolactate to diacetyl. In both the absence and presence of oxygen, this ratio was higher in the \(\alpha\)-acetolactate decarboxylase-negative mutant.

In the presence of oxygen, parental strain CNRZ 483 produced diacetyl and acetoin in parallel to citrate consumption. Diacetyl production by this strain could be the result of a chemical reaction involving spontaneous oxidative decarboxylation of a small quantity of \(\alpha\)-acetolactate produced by the cell. Strain CNRZ 483, however, exhibited no detectable transitory accumulation of \(\alpha\)-acetolactate. Considering the sensitivity of the assay method, the concentration of this compound was lower than 0.1 mM throughout fermentation. Since growth and acidification kinetics were similar in the two strains, the degree of oxidative and non oxidative spontaneous degradation of \(\alpha\)-acetolactate in cultures of the \(\alpha\)-acetolactate decarboxylase-negative mutant 483 M1 can be applied to the parental strain. The maximal quantity of \(\alpha\)-acetolactate that could have undergone a spontaneous decarboxylation in cultures of strain CNRZ 483 is thus equal to the product of the maximal \(\alpha\)-acetolactate concentration (0.1 mM), the rate of spontaneous degradation of \(\alpha\)-acetolactate (0.06 h\(^{-1}\)) and time (24 h). Thus, less than 0.14 mM \(\alpha\)-acetolactate could have been spontaneously degraded to diacetyl and acetoin when the parental strain was grown in the presence of oxygen. The spontaneous oxidative decarboxylation of the \(\alpha\)-acetolactate produced in the culture medium cannot explain the production of 0.7 mM diacetyl observed in our conditions. Diacetyl synthesis by the wild type strain might be explained by a high intracellular accumulation of \(\alpha\)-
acetolactate, which could be converted spontaneously into diacetyl inside of the cells. However, given that diacetyl and α-acetolactate were assayed after distillation of the culture medium containing the bacteria, the concentration of these compounds also takes account of a possible intracellular accumulation. Consequently, an intracellular accumulation of α-acetolactate couldn’t represent more than 0.1 mmol. in 1 litre of culture medium. Another possibility is that in wild type cells, diacetyl arises from an oxidative decarboxylation of α-acetolactate, which is catalysed (i.e. not spontaneous) and intracellular. Recently, Rondags et al. (21) demonstrated that in Lactococcus lactis, a thermolabile compound favoured the production of diacetyl from α-acetolactate. Oxido-reduction coenzymes might be involved in this conversion (19). Jordan et al. (13) have also established that a non-spontaneous oxidative decarboxylation of α-acetolactate occurred in cell-free extracts of Leuconostoc lactis. According to these authors, it is possible that α-acetolactate decarboxylase, the enzyme catalysing the decarboxylation of α-acetolactate into acetoain, also leads to the enzymatic production of small quantities of diacetyl from α-acetolactate.
ACKNOWLEDGMENTS

This research was supported by contract AIR3-CT94-2010 from the European Union.

The authors thank G. Yonnet and M.C. Piron for helpful technical assistance.
REFERENCES


TABLE 1. Growth and acidification characteristics of *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1, grown in milk at 30°C in different conditions

<table>
<thead>
<tr>
<th></th>
<th>CNRZ 483</th>
<th>483 M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of oxygen</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Addition of 14 mM citrate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Final A (405 nm)</td>
<td>5.45</td>
<td>5.62</td>
</tr>
<tr>
<td>Maximal growth rate (h⁻¹)</td>
<td>0.83</td>
<td>0.55</td>
</tr>
<tr>
<td>Maximal acidification rate (pH unit.h⁻¹)</td>
<td>-0.28</td>
<td>-0.15</td>
</tr>
<tr>
<td>pH at 24 h</td>
<td>4.33</td>
<td>5.04</td>
</tr>
</tbody>
</table>


TABLE 2. α-Acetolactate metabolism by *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1, grown in milk at 30°C, in different conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>CNRZ 483</th>
<th>483 M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of oxygen</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Addition of 14 mM citrate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diacetyl / (Diacetyl plus acetoin) (%)</td>
<td>&lt; 1.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Diacetyl plus acetoin plus α-acetolactate (mM)</td>
<td>2.1</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Values after 24 h of culture
FIG. 1. Pathway for diacetyl metabolism in *Lactococcus lactis*. Enzyme or step: 1, lactate dehydrogenase; 2, pyruvate dehydrogenase; 3, pyruvate formate lyase; 4, α-acetolactate synthase; 5, α-acetolactate decarboxylase or spontaneous non-oxidative decarboxylation; 6, spontaneous oxidative decarboxylation; 7, diacetyl/acetoin reductase.

FIG. 2. Changes of absorbance, pH and citrate concentration in cultures of *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1 grown in milk in different conditions: without oxygen (●), with oxygen (■), with oxygen and addition of 14 mM citrate (♦).

FIG. 3. Production of lactate and acetate by *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1 grown in milk in different conditions: without oxygen (●), with oxygen (■), with oxygen and addition of 14 mM citrate (♦).

FIG. 4. Production of diacetyl, acetoin and α-acetolactate by *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 and the α-acetolactate decarboxylase-negative mutant 483 M1 grown in milk in different conditions: without oxygen (●), with oxygen (■), with oxygen and addition of 14 mM citrate (♦).

FIG. 5. Diacetyl production as a function of citrate consumption in cultures of *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 (closed symbols) and α-acetolactate decarboxylase-negative mutant 483 M1 (open symbols). Strains were grown in milk in different conditions: without oxygen (●,♦), with oxygen (■,□), with oxygen and addition of 14 mM citrate (▲,△).
Fig. 3
Fig. 5