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**Selection and properties of α -acetolactate decarboxylase-deficient spontaneous mutants
of *Streptococcus thermophilus***

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Running title: alpha-acetolactate decarboxylase-deficient mutants

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Abstract

Many lactic acid bacteria produce diacetyl, which is a desirable aroma compound in some fermented dairy products. Strains or mutants of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* that are deficient in α -acetolactate decarboxylase are used in some food processes for their ability to produce large amounts of diacetyl. However, up until now, the use of α -acetolactate decarboxylase-deficient mutants of *Streptococcus thermophilus* for increased diacetyl production has not been evaluated. The objective of the present study was to devise a procedure for selecting spontaneous α -acetolactate decarboxylase-deficient mutants of *Streptococcus thermophilus*. We observed that in a chemically defined medium containing α -ketobutyrate plus leucine, or α -ketobutyrate plus leucine plus isoleucine, the α -acetolactate decarboxylase-deficient mutant TIL865, obtained by directed mutagenesis, grew faster than its parent strain. This property was used for selecting spontaneous α -acetolactate decarboxylase-deficient mutants on agar plates. The resulting mutants were able to grow in milk, and their acidifying activity was slightly lower than that of the parent strain. Under partial anaerobic or aerobic conditions, they produced approximately three times more diacetyl than the parent strain. Such spontaneous mutants may be useful for increasing the diacetyl content of fermented milks whose production involves *Streptococcus thermophilus* strains.

1. Introduction

Diacetyl is a major flavor compound in many cultured dairy products. It is an end product of citrate metabolism by certain lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* sp, which are used for the production of cultured cream, cultured butter, buttermilk and fresh cheeses. Diacetyl is also a key aroma compound in yogurt (Imhof et al., 1995; Ott et al., 1997), but in this case, it is mainly produced by *Streptococcus thermophilus*, which is a lactic acid bacterium that is not able to use citrate.

Strains of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* that are deficient in α -acetolactate decarboxylase, the enzyme responsible for acetoin production, accumulate significant amounts of α -acetolactate in the culture medium. This results in higher diacetyl production due to the spontaneous oxidative decarboxylation of α -acetolactate (De Man, 1959). Such strains are widely used in butter-making processes (Veringa et al., 1976) and for the production of aroma additives with a high α -acetolactate (Kuiper et al., 1987) or diacetyl (Jönsson et al., 1980) content. In *Lactococcus lactis* strains isolated from non-dairy environments, α -acetolactate decarboxylase is also involved in the regulation of leucine and valine biosynthesis (Goupil-Feuillerat et al., 1997). Indeed, α -acetolactate is a precursor of these branched-chain amino acids, and when they are present in excess, the flux of α -acetolactate is diverted towards acetoin, due to an allosteric activation of the α -acetolactate decarboxylase (Phalip et al., 1994) and to a translational regulation of α -acetolactate decarboxylase synthesis (Goupil-Feuillerat et al., 2000). Goupil and co-workers (1996) took advantage of the fact that this enzyme has a central role in the regulation of branched-chain amino acid biosynthesis to select spontaneous α -acetolactate decarboxylase-deficient mutants, by using a defined medium containing leucine, but devoid of valine and isoleucine.

We recently showed that α -acetolactate decarboxylase is also involved in the regulation of leucine and valine biosynthesis by *Streptococcus thermophilus* (Monnet et al., 2003) (Fig. 1). The objective of the present study was to determine whether this property could be used for devising a medium for the selection of spontaneous α -acetolactate decarboxylase-deficient mutants, and whether such mutants were able to produce large amounts of diacetyl in milk.

2. Materials and Methods

2.1. Bacterial strains

Streptococcus thermophilus strains were routinely grown in M17 broth (Terzaghi and Sandine, 1975). Strain CNRZ385 was obtained from UBLO (Unité Bactéries Lactiques et Pathogènes Opportunistes, INRA, Jouy-en-Josas, France), and strain TIL865 is an α -acetolactate decarboxylase-deficient mutant selected from strain CNRZ385 by directed mutagenesis (Monnet et al., 2003).

2.2. Growth in defined media

The effect of branched-chain amino acids and α -ketobutyrate on the growth of *Streptococcus thermophilus* was studied using a chemically defined medium (CDM), derived from the medium described by Reiter and Oram (1962). It contained lactose (10 g/l), KH_2PO_4 (3 g/l), sodium acetate (1 g/l), ascorbic acid (0.5 g/l), adenine (5 mg/l), guanine (5 mg/l), xanthine (5 mg/l), uracil (5 mg/l), pyridoxal hydrochloride (2 mg/l), niacin (1 mg/l), thiamine

95 hydrochloride (1 mg/l), riboflavin (1 mg/l), calcium pantothenate (1 mg/l), para-aminobenzoic
96 acid (10 µg/l), biotin (10 µg/l), folic acid (1 µg/l), vitamin B12 (1 µg/l), MgCl₂.6H₂O (200
97 mg/l), CaCl₂ (50 mg/l), FeCl₃.6H₂O (5 mg/l), ZnSO₄.7H₂O (5 mg/l), CoCl₂.6H₂O (2.5 mg/l),
98 CuSO₄.5H₂O (2.5 mg/l), NiSO₄.7H₂O (10 mg/l), MnSO₄.1H₂O (10 mg/l), L-cysteine
99 hydrochloride (200 mg/l), L-alanine (150 mg/l), L-lysine hydrochloride (800 mg/l), L-
100 arginine (450 mg/l), L-proline (540 mg/l), L-methionine (70 mg/l), L-phenylalanine (100
101 mg/l), L-serine (200 mg/l), L-threonine (100 mg/l), L-tryptophane (100 mg/l), glycine (200
102 mg/l), L-histidine hydrochloride.1H₂O (550 mg/l), L-glutamate (3.2 g/l), L-glutamine (3.2
103 g/l), L-aspartate (300 mg/l) and L-tyrosine (100 mg/l). The medium was reconstituted using
104 concentrated stock solutions containing the nutrients. A mixture containing lactose, salts and
105 vitamins was prepared at a concentration double that in the CDM. After adjusting its pH to
106 6.8 using NaOH, the mixture was autoclaved for 15 min at 110°C. The amino acids were
107 prepared as a five times concentrated solution that was filter-sterilized (0.22 µm) after
108 adjustment of its pH to 6.8. Supplementation of CDM with L-leucine, L-isoleucine, L-valine
109 or sodium α-ketobutyrate was done at a final concentration of 200, 100, 150 and 600 mg/l,
110 respectively. These compounds were prepared individually as 15 times concentrated solutions
111 that were filter-sterilized (0.22 µm) after adjusting their pH to 6.8. After two successive
112 cultures in CDM at 37°C, cells of *Streptococcus thermophilus* were recovered by
113 centrifugation for 10 min at 5,000 x g and 4°C, washed in a saline solution (9 g/l NaCl), and
114 resuspended in the same solution to reach an absorbance (575 nm) equivalent to 1.4. Cells
115 were then inoculated at 0.5% (v/v) in CDM. Cultures were performed at 37°C, under partial
116 anaerobic conditions (incubation without agitation, where the headspace volume represented
117 less than 2% of the culture volume).

118

Spontaneous mutants of *Streptococcus thermophilus* deficient in α -acetolactate decarboxylase were selected using an agar medium, which was prepared by supplementing CDM with agar (15 g/l), L-leucine (200 mg/l) and sodium α -ketobutyrate (600 mg/l).

2.3. Milk cultures

Cells were grown at 37°C in M17 broth and harvested at the end of exponential growth phase. The density of the cell suspension in the broth was estimated by its absorbance at 575 nm. Cells were washed with 0.9% NaCl and inoculated, at a concentration equivalent to 0.02 absorbance unit, in reconstituted skim milk (100 g/l; Epi-Ingredients, Ancenis, France) that had been heated for 10 min at 110°C. Static cultures (partial anaerobiosis) were grown in 250-ml conical flasks containing 200 ml of milk at 37°C. The pH electrodes, disinfected with ethanol, were introduced through bores in the rubber stoppers. The pH was continuously measured using a CINAC apparatus (Alliance Instruments, Frépillon, France). Aerobic cultures were grown in 250-ml conical flasks containing 100 ml of milk and were incubated at 37°C on a rotary shaker at 140 rpm.

2.4. Measurement of diacetyl, acetoin and α -acetolactate concentration

Diacetyl and α -acetolactate were determined as described by Mohr et al. (1997). The sum of diacetyl and acetoin was determined by the method of Westerfeld (1945), as modified by Monnet et al. (1994), and acetoin was calculated by subtracting the diacetyl concentration.

2.5. Measurement of α -acetolactate decarboxylase activity

At the end of the exponential growth phase, *Streptococcus thermophilus* cells were harvested by centrifugation of 250 ml of culture for 15 min at 14,000 x g and 4°C. They were then washed with 250 ml of sodium phosphate buffer (50 mM, pH 6), resuspended in 1.5 ml of the same buffer, and placed in a 2-ml tube containing 600 mg of 0.1 mm diameter glass beads (PolyLabo, Strasbourg, France). They were then disrupted in a cell disruptor (FP120 FastPrep™; Savant Instruments Inc., Holbrook, USA) for 30 s at 4°C and at speed 6.5. After centrifugation for 5 min at 21,000 x g and 4°C, α -acetolactate decarboxylase activity was determined in the supernatant (Løken and Størmer, 1970). The reaction mixture contained sodium phosphate buffer (200 mM, pH 6), valine (100 mM) and cell-free extract. The reaction was started by adding 120 mM of α -acetolactate. This compound was generated by hydrolyzing ethyl 2-acetoxy-2-methyl-acetoacetate (Aldrich, Saint-Quentin-Fallavier, France) with two equivalents of NaOH (Veringa et al., 1984). The reaction was carried out at 20°C for 30 min, and the resulting solution was immediately used for the assay of α -acetolactate decarboxylase. Acetoin production from α -acetolactate was determined with the method of Westerfeld (1945), which is based on a spectrophotometric measurement at 530 nm. Since α -acetolactate undergoes a slow non-enzymatic decarboxylation into acetoin or diacetyl, compounds that give rise to complexes absorbing at 530 nm, a control reaction was run without cell-free extract. One enzyme unit was defined as 1 μ mol of acetoin produced per min at 37°C. Protein concentration in cell-free extracts was determined with the method of Bradford (1976), using bovine serum albumin as a protein standard.

2.6. Sequencing of the *aldC* gene from *Streptococcus thermophilus* mutants

The *aldC* gene (1.6 kb) was PCR-amplified using primers ald4 (5'-GAC ATC AAA CGC TTG GGC-3') and ald5 (5'-TGG TGC CTC ATC AGA AGC-3'). Thermostable *Taq*

DNA polymerase, buffer and dNTP mixture were purchased from Takara Biomedicals (Shiga, Japan). The concentration of primers, *Taq* DNA polymerase, dNTPs and MgCl₂ in the reaction mixture was 0.6 μM, 0.025 U/μl, 0.2 mM and 2 mM, respectively. *Streptococcus thermophilus* genomic DNA was prepared as described by Pospieh and Neumann (1995), and added to the PCR mixture at a concentration of 0.1 ng/μl. After initial denaturation (5 min at 94°C) and 25 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 52°C) and elongation (2 min at 72°C), a final incubation for 5 min at 72°C was performed. The resulting amplicon was sequenced by Genome Express (Meylan, France), using primers ald4 and ald8 (5'-GGT TGA CTT TGG TCC TGT-3'). Each strand was sequenced twice using independent PCR amplicons. The sequences were then assembled using the CAP2 program (Huang, 1996) and compared using the Pretty program of SeqWeb (Version 2) of the Genetics Computer Group (Accelrys, Inc., San Diego, CA, USA).

2.7. Statistical analyses

Comparisons of the means of growth, acidifying activity and production of α-acetolactate and its decarboxylation products were done using a Student's test.

3. Results

3.1. Comparison of growth of *Streptococcus thermophilus* CNRZ385 and its α-acetolactate decarboxylase-deficient mutant TIL865 in defined media

In *Lactococcus lactis* strains isolated from non-dairy environments, the α-acetolactate decarboxylase is involved in the regulation of leucine and valine biosynthesis. Goupil and co-

workers (1996) showed that this regulation explains that only α -acetolactate decarboxylase-deficient strains of *Lactococcus lactis* are able to grow in a defined medium containing leucine, but devoid of valine and isoleucine, and that this property can be used for selecting spontaneous mutants. In order to determine whether a similar medium can be devised for the selection of α -acetolactate decarboxylase-deficient mutants of *Streptococcus thermophilus*, we compared the growth of strain CNRZ385 and its α -acetolactate decarboxylase-deficient mutant TIL865 in various defined media (Table 1). In contrast to what is observed in *Lactococcus lactis*, *Streptococcus thermophilus* CNRZ385 is able to grow in a defined medium containing leucine, but devoid of valine and isoleucine. Its growth is even slightly higher than that of the α -acetolactate decarboxylase-deficient mutant TIL865. However, we observed that when the defined medium contains α -ketobutyrate (a precursor of isoleucine biosynthesis) and is devoid of valine, growth of the parent strain is much lower than that of the mutant in the presence of leucine or of leucine plus isoleucine (Table 1).

3.2. Selection and properties of spontaneous α -acetolactate decarboxylase-deficient mutants

The inhibitory effect of leucine plus α -ketobutyrate on the growth of the parent strain could also be observed on agar plates. Indeed, after 48 h of growth at 37°C, the colonies of parent strain were smaller (diameter less than 1 mm) than those of TIL865 (diameter approximately 4 mm). In order to determine whether spontaneous mutants could be selected on such an agar medium, a cell suspension of the parent strain CNRZ385 was inoculated at a concentration equivalent to 10^7 cells per agar plate. Under these conditions, each agar plate contained, in addition to numerous small colonies, approximately 20 large colonies. Five of these large colonies were selected at random, purified on M17 agar, and the corresponding clones were designated as 385-M1 to 385-M5.

219

220 After growth on liquid CDM supplemented with leucine plus isoleucine plus valine,
221 no α -acetolactate decarboxylase activity could be detected in cell-free extracts of any of the
222 five clones (< 0.01 U/mg of protein), whereas the activity of the parent strain was 0.32 ± 0.03
223 U/mg of protein. The five mutants were considered to be stable since no increase of α -
224 acetolactate decarboxylase activity could be detected when they were subcultivated ten times
225 at an inoculation ratio of 1% (equivalent to 66 generations).

226

227 As expected, mutations were present in the *aldC* gene (encoding α -acetolactate
228 decarboxylase) of the five mutants (Table 2). In mutants 385-M4 and 385-M5, the mutation
229 corresponded to a frame shift (the same for both of the mutants). In the next two mutants, a
230 stop codon was introduced into *aldC*. In the fifth mutant, an amino acid was replaced by
231 another.

232

233 The parent strain, the α -acetolactate decarboxylase-deficient mutant resulting from
234 directed mutagenesis, and the five spontaneous mutants were cultivated in milk under partial
235 anaerobic or aerobic conditions (Table 3). Under partial anaerobic conditions, the acidifying
236 activity of all mutants was slightly lower than that of the parent strain. Indeed, the time
237 required to reach pH 4.8 was approximately 20 to 30 min longer. The parent strain mainly
238 produced acetoin, which represented 90% of the cumulated production of diacetyl, acetoin
239 and α -acetolactate. As a consequence of the absence of α -acetolactate decarboxylase activity,
240 the mutants produced significant amounts of α -acetolactate (equivalent to 51 to 65% of the
241 cumulated production of diacetyl, acetoin and α -acetolactate), and their diacetyl production
242 was greater than that of the parent strain. Similar results were observed under aerobic
243 conditions, except that the production of diacetyl, acetoin and α -acetolactate was stimulated.

The concentration of diacetyl in mutant cultures was approximately three times higher than in cultures of the parent strain. No significant difference could be observed between the cultures of the spontaneous mutants and the culture of the mutant resulting from directed mutagenesis ($p < 0.05$).

4. Discussion

In the present study, we observed that, in contrast to *Lactococcus lactis* strains isolated from non-dairy environments, no inhibition of growth of *Streptococcus thermophilus* occurred in a defined medium containing leucine, but devoid of valine and isoleucine. This shows that the regulation of branched-chain amino acid biosynthesis in these bacteria is controlled differently. Growth of *Streptococcus thermophilus* is considerably reduced when the medium is supplemented with α -ketobutyrate (a precursor of isoleucine biosynthesis; Fig. 1). For the α -acetolactate decarboxylase-deficient mutant, the inhibition by α -ketobutyrate is less important when the medium is supplemented with leucine or with leucine plus isoleucine. Consequently, it is possible to select spontaneous α -acetolactate decarboxylase-deficient mutants on an agar medium that is composed of a CDM containing α -ketobutyrate and leucine.

The reason why the growth of the parent strain is much lower than that of the α -acetolactate decarboxylase-deficient mutants in the medium supplemented with α -ketobutyrate plus leucine, or with α -ketobutyrate plus leucine plus isoleucine is not known. Nevertheless, it is interesting to observe that α -ketobutyrate inhibits the growth of the parent strain, except when valine is present in the medium (Table 1). Therefore, the observed reduced growth may result from a limitation in valine biosynthesis. It is thus possible that in

the presence of α -ketobutyrate plus leucine or of α -ketobutyrate plus leucine plus isoleucine, the synthesis of valine is limited, and the absence of α -acetolactate decarboxylase could then favor valine biosynthesis from α -acetolactate, resulting in a growth advantage for the mutants. However, it is well known that there are numerous genetic and enzymatic regulation mechanisms of branched-chain amino acid biosynthesis, and the explanation for the difference in behavior between the parent and the mutant strains may thus be much more complicated.

We observed that the suppression of α -acetolactate decarboxylase activity in *Streptococcus thermophilus* results in a higher diacetyl production, just like for *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Under our culture conditions, this production increased approximately three times. The spontaneous mutants were stable (over more than 66 generations), and mutations were present within the *aldC* gene. During growth in milk, the spontaneous mutants expressed the same behavior as the mutant resulting from directed mutagenesis, indicating that they probably did not contain detrimental nonspecific mutations (*i.e.*, other than those affecting α -acetolactate decarboxylase activity). The acidifying activity of the mutants was lower than that of the wild-type strain. However, the difference was not big, and this would probably not preclude the utilization of the mutants in industrial processes.

Diacetyl, acetoin and α -acetolactate production increased under aerobic conditions, both for the wild-type and the mutant strains. One possible explanation for that effect is that some NADH is then reoxidized to NAD by the NADH oxydase, leaving more pyruvate available for enzymatic reaction pathways that do not consume NADH, such as the α -acetolactate synthase pathway (acetoin production). Moreover, the increase of diacetyl

294 production in the presence of oxygen also results from a stimulation of the spontaneous
295 oxidative decarboxylation of α -acetolactate (De Man, 1959).

296

297 In summary, the present study shows that it is easy to select α -acetolactate
298 decarboxylase-deficient spontaneous mutants of *Streptococcus thermophilus*. Such mutants
299 are able to grow in milk and to produce greater amounts of diacetyl. They may be useful for
300 increasing the diacetyl content of fermented milks whose production involves *Streptococcus*
301 *thermophilus* strains.

302

Table 1

Comparison of growth of the wild-type strain *Streptococcus thermophilus* CNRZ385 and its α -acetolactate decarboxylase-deficient mutant TIL865 in chemically defined media

Compounds present in the CDM				Absorbance (at 575 nm) after 15 h of growth ^a	
Leucine	Isoleucine	Valine	α -Ketobutyrate	CNRZ385	TIL865
				0.98	1.03
+				1.00	0.86*
	+			0.93	0.58*
		+		1.06	0.98
+	+			1.01	0.89*
+		+		1.00	0.91*
	+	+		1.03	1.08
+	+	+		1.16	1.11
			+	0.28	0.35*
+			+	0.14	0.70*
	+		+	0.12	0.12
		+	+	1.05	0.79*
+	+		+	0.13	0.69*
+		+	+	1.08	0.86*
	+	+	+	0.99	0.95
+	+	+	+	1.11	1.06

^a Values are means of three repetitions

* Values differed from those of the wild-type strain grown under the same conditions (p<0.05)

313 Table 2
 314 Mutations present in the *aldC* gene (encoding α -acetolactate decarboxylase) of the mutants
 315

Mutant	Differences in nucleotide sequence ^a	Consequence on <i>aldC</i> gene
385-M1	C replaced by A in position 470	Substitution of the serine codon 167 with a stop codon
385-M2	G replaced by T in position 365	Substitution of the glycine codon 122 with a valine codon
385-M3	G replaced by T in position 196	Substitution of the glutamine codon 66 with a stop codon
385-M4	Deletion of G in position 64	Frame shift
385-M5	Deletion of G in position 64	Frame shift

316
 317 ^a Sequences were compared to that of the parent strain (Genbank n°AY072795)
 318
 319

Table 3

Comparison of acidifying activity and production of α -acetolactate and its decarboxylation products, by the wild-type strain *Streptococcus thermophilus* CNRZ385, the α -acetolactate decarboxylase-deficient mutant resulting from directed mutagenesis TIL865, and the spontaneous mutants 385-M1 to 385-M5^a

Strain	Partial anaerobic conditions				Aerobic conditions			
	tpH _{4.8} ^b (h)	Diacetyl (mM)	Acetoin (mM)	Acetolactate (mM)	tpH _{4.8} (h)	Diacetyl (mM)	Acetoin (mM)	Acetolactate (mM)
CNRZ385	3.84	0.015	0.517	0.038	4.91	0.071	1.312	0.095
TIL865	4.21*	0.033*	0.100*	0.245*	5.79*	0.244*	0.187*	0.679*
385-M1	4.27*	0.034*	0.100*	0.192*	5.72*	0.214*	0.189*	0.743*
385-M2	4.25*	0.029*	0.109*	0.248*	5.74*	0.235*	0.173*	0.739*
385-M3	4.23*	0.028*	0.099*	0.233*	5.60*	0.234*	0.190*	0.759*
385-M4	4.18*	0.038*	0.091*	0.205*	5.83*	0.197*	0.212*	0.787*
385-M5	4.31*	0.044*	0.109*	0.162*	5.68*	0.193*	0.219*	0.737*

^a Cultures were analyzed at pH 4.8

^b Time required to reach pH 4.8

* Values differed from those of the wild-type strain grown under the same conditions (p<0.05)

Fig. 1. Schematic representation of the pathways involved in diacetyl and branched-chain amino acid biosynthesis by *Streptococcus thermophilus*. Continuous arrows indicate enzymatic steps, dotted double arrows indicate non-enzymatic steps, and dotted simple arrows indicate allosteric activations. ALS, α -acetolactate synthase; AHAS, acetohydroxy acid synthase; ALDC, α -acetolactate decarboxylase; COD, chemical oxidative decarboxylation; CNOD, chemical non-oxidative decarboxylation.

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