Selection and Properties of *Lactobacillus* Mutants Producing α-Acetolactate

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ABSTRACT

Many lactobacilli produce diacetyl, a desirable aroma in some fermented dairy products. The objective of the present work was to obtain mutants of lactobacilli that were deficient in α-acetolactate decarboxylase and to determine whether they had improved aroma-producing activity. Nine mutants of *Lactobacillus rhamnosus* that were deficient in α-acetolactate decarboxylase were selected by screening for colonies that produced α-acetolactate from pyruvate on agar plates. When grown in milk, only one-third of the mutants exhibited the same acidification behavior as the parent strain. In addition, even though α-acetolactate was produced by all of the mutants, none of them produced more diacetyl than did the parent strains. This result shows that the suppression of α-acetolactate decarboxylase activity does not cause increased production of diacetyl by all lactic acid bacteria. Such a strategy would be interesting only for parent strains producing large quantities of acetoin (e.g., citrate-utilizing *Lactococcus lactis* ssp. *lactis*)

(**Key words**: *Lactobacillus*, α-acetolactate, diacetyl, α-acetolactate decarboxylase)

Abbreviation key: **Cit$^+$** = citrate-utilizing, **LAB** = lactic acid bacteria, **NTG** = N-methyl-N′-nitro-N-nitrosoguanidine.

INTRODUCTION

Lactobacilli are used in the manufacture of several kinds of fermented milks and fresh and ripened cheeses. These bacteria are sought for their aromatic, acidifying, proteolytic, and texturizing properties as well as for their probiotic role. Many aromas produced by lactobacilli result from the reaction of α-dicarbonyl compounds with amines (10). Reps et al. (22) showed that *Lactobacillus delbrueckii* ssp. *bulgaricus* could produce glyoxal, methylglyoxal, and diacetyl. Diacetyl is also synthesized by lactic acid bacteria (**LAB**) of the genus *Leuconostoc* and citrate-utilizing (**Cit$^+$**) *Lactococcus lactis* ssp. *lactis*. The diacetyl aroma is characteristic of many fresh dairy products, such as buttermilk, butter, and some fresh cheeses. Considerable work has been devoted to understanding the synthesis of diacetyl by LAB. In the presence of excess pyruvate, α-acetolactate synthase condenses two molecules of pyruvate to form CO_2 and α-acetolactate. The latter is unstable, spontaneously decarboxylating to form diacetyl in the presence of oxidizing conditions. In a reducing medium, decarboxylation of α-acetolactate yields primarily acetoin, a nonaromatic compound. Furthermore, LAB possess an α-acetolactate decarboxylase that transforms α-acetolactate to acetoin, which explains why aroma-producing LAB produce much more acetoin than diacetyl even in the presence of oxygen. Several Cit$^+$ *L. lactis* ssp. *lactis* strains lack α-acetolactate decarboxylase and have the ability to accumulate α-acetolactate in the medium, which results in the production of large quantities of diacetyl, particularly under aerobic conditions (17). The starter culture 4/25, which produces great amounts of diacetyl and is used in butter making, contains a naturally occurring Cit$^+$ *L. lactis* ssp. *lactis* strain that is negative for α-acetolactate decarboxylase (12, 13, 25). Isolation of α-acetolactate decarboxylase-negative mutants is, thus, of considerable interest. The disruption of this enzyme by genetic engineering has been described for *L. lactis* ssp. *lactis* (8). Goupil et al. (9) proposed a method for the selection of spontaneous mutants that could be applied to *L. lactis* ssp. *lactis* strains that were prototrophic for isoleucine and valine. Another method has been described that is based on random mutagenesis followed by screening for clones that accumulate α-acetolactate (18).

Despite these descriptions, very little work has been devoted to obtaining mutants of lactobacilli with intense aroma production. Bednarski and Hammond (2) devised a method for the detection of *Lactobacillus* spp. mutants producing quantities of α-dicarbonyl compounds that were different from those of their

Received December 22, 1997.
Accepted April 15, 1998.
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parents, but the type of mutations that was created in these strains was not investigated. Montville et al. (20) obtained *Lactobacillus plantarum* mutants that produced increased amounts of acetoin.

Strains of *Lactobacillus rhamnosus* are sometimes used in the manufacture of fermented milks. The objective of the present work was to obtain mutants of *Lb. rhamnosus* that were deficient in α-acetolactate decarboxylase and to investigate the production of α-acetolactate and diacetyl during the bacterial growth in milk.

MATERIALS AND METHODS

Strains

Lactobacillus rhamnosus CNRZ 212 was obtained from the collection of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France), and *Lb. rhamnosus* F414 was obtained from the Laboratoire de Génie et Microbiologie des Procédés Alimentaires (Thiverval-Grignon, France). Both strains were routinely propagated in MRS broth (7) at 37°C.

Obtaining Mutants

Cells were grown at 37°C in 5 ml of MRS broth and harvested by centrifugation at the end of the exponential growth phase. The cells were washed with 100 m*M* potassium phosphate buffer (pH 7) and resuspended in 0.5 ml of the buffer. A volume of 0.5 ml of N-methyl-N′-nitro-N-nitrosoguanidine (**NTG**) solution was then added, and the suspension was incubated for 1 h at 37°C. The cells were harvested by centrifugation, washed with 5 ml of buffer, and resuspended in 5 ml of MRS broth. The suspension was incubated for 1 h at 37°C to resuscitate the cells. The culture was then mixed by vortexing for 2 min, diluted to obtain approximately 60 colonies per plate, and inoculated onto 200 agar plates (90-mm diameter) of screening medium. The screening medium was prepared as previously described (18), except that it was supplemented with Tween 80 (1 ml/L) and citrate was replaced by sodium pyruvate (5.5 g/L). After an incubation of 60 h at 30°C, the agar plates were screened for colonies producing α-acetolactate (18). API 50 CH strips (BioMérieux, Marcy l'Etoile, France) were used to verify that the selected mutants were not contaminants.

Culture Conditions

Cells were grown at 37°C in MRS broth and were harvested at the end of the exponential growth phase. The density of the cell suspension in the broth was estimated by its absorbance at 575 nm. Cells were washed twice with 0.9% NaCl and inoculated in UHT-sterilized skim milk (Candia, Lyon, France) at a concentration equivalent to 0.02 absorbance unit. 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 and led to the determination of the maximum acidification rate (change in pH per min) (23). Aerobic cultures were grown in 2-L conical flasks containing 500 ml of milk and were incubated at 37°C on a rotary shaker at 200 rpm.

Bacterial Counts and Analytical Methods

Samples for bacterial counts were diluted (1:100, vol/vol) in 0.9% NaCl and dispersed with a mechanical blender (Ultra-Turrax® model T25; Ika Labortechnik, Stafen, Germany) for 30 s. The suspensions were then dispensed on MRS agar plates using a Spiral system (Interscience, Saint Nom la Breteche, France), and colonies were counted after the agar plates had been incubated for 3 d at 37°C.

Diacetyl and α-acetolactate were determined as described by Mohr et al. (15). The sum of diacetyl and acetoin was determined by the method of Westerfeld (26), as modified by Monnet et al. (17), and acetoin was calculated by subtracting the diacetyl concentration. Citrate was determined with an enzymatic method (Boehringer Mannheim, Mannheim, Germany), and 2,3-butanediol was determined by HPLC as described by Bassit et al. (1).

Assays of Enzymatic Activities

Cells that were cultivated at 37°C in 1 L of MRS broth were harvested at the end of exponential growth phase by centrifugation at 14,000 × *g* at 4°C for 15 min. After cells were washed with 200 ml of 50 m*M* sodium phosphate buffer (pH 7), the bacteria were resuspended (1:20, vol/vol) in buffer and treated with a sonicator (Sonifier® II; Branson ultrasonic, Carouge-Geneva, Switzerland) for 5 × 1 min at 0°C and 50 W. After another centrifugation (25,000 × *g* for 30 min), enzymatic activities were determined in the supernatant. Lactate dehydrogenase was measured by monitoring the decrease in absorbance at 340 nm. The assay was performed in 50 m*M* acetate buffer (pH 5.4) containing 10 m*M* sodium pyruvate, 1 m*M* fructose-1,6-diphosphate, 1 m*M* $MnCl_2$, and 0.15 m*M* NADH (11). Acetoin and diacetyl reductase were measured as described by Cogan (6). These enzyme activities were corrected for NADH oxidase

activities, determined in the same conditions but in the absence of substrate (pyruvate, diacetyl, and acetoin for lactate dehydrogenase, diacetyl reductase, and acetoin reductase, respectively). The NADH oxidase activities reported (Table 1) are those in the diacetyl reductase assay. These NADH oxidase activities were slightly different from those in the lactate dehydrogenase assay. One enzyme unit was defined as 1 μmol of NADH oxidized/min. α-Acetolactate synthase activity was determined by measuring the acetoin production from pyruvate (6). The assay was performed in 100 m*M* sodium phosphate buffer (pH 6.5) containing 80 m*M* sodium pyruvate and 0.21 m*M* thiamine pyrophosphate. After 15 min of incubation at 45°C, HCl was added at a final concentration of 0.1 *M* to stop the reaction and to convert α-acetolactate to acetoin. After 30 min of incubation at 45°C, the acetoin that formed was measured with the colorimetric method of Westerfeld (26). α-Acetolactate decarboxylase activity was measured at pH 6.0 as previously described (16), except that the D-α-acetolactate concentration was adjusted to 5 m*M*. One unit of α-acetolactate synthase (α-acetolactate decarboxylase) activity was defined as the formation of 1 μmol of α-acetolactate (acetoin)/min. Protein concentrations were determined with the method of Bradford (5) using BSA as a protein standard.

RESULTS

Selection of Mutants

The screening of Cit$^+$ *L. lactis* ssp. *lactis* mutants, as described previously (18), was conducted in the presence of a high concentration of citrate in order to maximize the production of α-acetolactate by mutants. This method was not directly applicable to *Lb. rhamnosus* CNRZ 212 and F414 because these strains are poor citrate utilizers. However, in the presence of pyruvate, many species of lactobacilli produce high quantities of acetoin (3, 4, 19, 24). Under such conditions, mutants for which α-acetolactate decarboxylase activity was inactivated can probably accumulate α-acetolactate from pyruvate, which is why the screening medium described previously (18) was modified to replace citrate by 50 m*M* sodium pyruvate.

The effect of the NTG concentration on cell survival during mutagenesis was determined. A survival level close to 10% by CNRZ 212 and F414 strains was obtained with NTG at 200 μg/ml, the concentration used to screen for mutants producing α-acetolactate. In all, we screened 7500 colonies arising from *Lb. rhamnosus* CNRZ 212 treated with NTG. Among them, 2 colonies were selected (clones 212M1 and 212M2) because they were surrounded by a red halo. Seven presumed mutants of strain F414 (clones 414M1 to 414M7) were selected during the screening of 9500 colonies. The percentage of colonies selected during the screening of strains CNRZ 212 and F414 was 0.03 and 0.07%, respectively.

Enzymatic Activities

The activities of six enzymes involved in pyruvate metabolism and the synthesis of α-acetolactate and diacetyl were assayed to determine the nature of the mutations in the clones selected after screening (Ta-

TABLE 1. Comparison of enzymatic activities in the cell-free extracts of *Lactobacillus rhamnosus* CNRZ 212 and F414 and mutants selected from these strains.

Parent strain and mutant	Specific activities[1]					
	α-Acetolactate decarboxylase	α-Acetolactate synthase	Lactate dehydrogenase	NADH Oxidase	Diacetyl reductase	Acetoin reductase
	(U/mg of protein)					
CNRZ 212	1.56	0.112	37.7	2.76	0.15	0.18
212M1	<0.01	0.091	38.5	2.46	0.20	0.29
212M2	0.05	0.119	36.5	2.49	0.20	0.29
F414	1.53	0.036	30.8	0.26	<0.01	<0.01
414M1	<0.01	0.038	21.5	0.16	<0.01	<0.01
414M2	<0.01	0.022	26.8	0.05	<0.01	<0.01
414M3	<0.01	0.029	20.7	0.07	<0.01	<0.01
414M4	<0.01	0.029	17.1	0.19	<0.01	<0.01
414M5	<0.01	0.034	26.3	0.18	<0.01	<0.01
414M6	<0.01	0.033	33.1	0.38	<0.01	<0.01
414M7	<0.01	0.038	16.3	0.25	<0.01	<0.01

[1]The values are means of at least three measurements with the same cell-free extract. The standard error was lower than 10% for all enzymes, except for α-acetolactate decarboxylase, diacetyl, and acetoin reductase, for which standard error was lower than 20%.

TABLE 2. Comparison of growth and acidification characteristics of milk by *Lactobacillus rhamnosus* CNRZ 212 and F414 and the mutants selected from these strains.[1,2]

Parent strain and mutant	Viable cells[3]		V_M[4]		pH_{24}	
	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$
	(cfu/ml)		(ΔpH/min)			
CNRZ 212	2.12×10^9	4.88×10^8	0.0020	ND[5]	4.76	5.84
212M1	2.15×10^9	4.57×10^8	0.0014*	ND	5.08*	5.75
212M2	1.83×10^9	4.37×10^8	0.0019	ND	4.86	6.02
F414	2.89×10^9	5.33×10^8	0.0030	ND	4.41	5.85
414M1	1.33×10^9*	3.12×10^8*	0.0020*	ND	5.89*	6.10*
414M2	3.03×10^8*	2.40×10^7*	0.0014*	ND	5.48*	5.99
414M3	2.10×10^9	6.03×10^8	0.0026	ND	4.75	5.89
414M4	9.33×10^8*	2.12×10^8*	0.0016*	ND	5.39*	6.06*
414M5	3.53×10^8*	2.89×10^8*	0.0014*	ND	5.87*	6.05*
414M6	2.39×10^9	5.71×10^8	0.0026	ND	4.48	5.78
414M7	5.73×10^8*	2.00×10^8*	0.0017*	ND	5.19*	5.79

[1]Cultures were grown under static ($-O_2$) or under aerobic ($+O_2$) conditions at 37°C.

[2]Means of three experiments.

[3]Cell concentration after 24 h of culture.

[4]V_M = Maximum acidification rate, and pH_{24} = pH after 24 h of culture.

[5]Not determined.

*Values differed from those of the parent strain grown in the same conditions ($P < 0.05$).

ble 1). Preliminary experiments showed that α-acetolactate decarboxylase in the two parent strains was not activated by branched-chain amino acids and that the substrate concentration (5 m*M*) used to measure the activities was sufficient to saturate the enzyme. As expected, the mutants selected had no α-acetolactate decarboxylase activity, except for mutant 212M2, which had residual activity of 3% that of the parent strain. The α-acetolactate synthase activities of strains CNRZ 212 and F414 were much lower than those of α-acetolactate decarboxylase, but these activities were not seriously affected by mutagenesis. Differences in lactate dehydrogenase, NADH oxidase, and diacetyl and acetoin reductase between the parent and the mutants were only slight. No diacetyl or acetoin reductase activities were detected in *Lb. rhamnosus* F414 or its mutants.

Growth and Acidification Activity

After 24 h of static culture in milk, the cell density of mutants 212M1 and 212M2 was similar to that of the parent strain, approximating 2×10^9 cfu/ml (Table 2). Cell numbers of *Lb. rhamnosus* F414 were 2.89×10^9 cfu/ml after 24 h of growth, and only the mutants 414M3 and 414M6 reached a comparable value. Agitation of the cultures (aerobic conditions) led to a considerable decrease in the growth of all of the strains.

Two acidification characteristics were determined in static cultures: the maximal rate of acidification and the pH after 24 h of culture (Table 2). The acidification characteristics of mutant 212M2 were similar to those of the parent strain; however, strain 212M1, with a lower maximal rate of acidification, had a higher pH after 24 h of culture than did *Lb. rhamnosus* CNRZ 212. Of the 7 mutants of strain F414, only 414M3 and 414M6 had acidification characteristics similar to that of the parent strain. For the other 5 mutants, the maximal rate of acidification was lower, and the value of the pH after 24 h of culture was higher. Agitation of the cultures led to an increase of the final pH values for all of the strains (Table 2). The decreased acidification activity of part of the mutants in static or aerobic cultures cannot be explained by the absence of α-acetolactate decarboxylase activity. The α-acetolactate decarboxylase activity of mutants 212M2, 414M3, and 414M6 was absent or highly attenuated, and their acidification activities were not significantly changed by the mutagenesis treatment.

Production of Diacetyl, Acetoin, and α-Acetolactate

After 24 h of static culture in milk, *Lb. rhamnosus* CNRZ 212 produced 1.35 m*M* of acetoin, but no α-acetolactate production could be detected (Table 3). Mutants 212M1 and 212M2 produced approximately

TABLE 3. Comparison of the production of diacetyl, acetoin, and α-acetolactate in milk by *Lactobacillus rhamnosus* CNRZ 212 and F414 and the mutants selected from these strains.[1,2]

Parent strain and mutant	Diacetyl		Acetoin		α-Acetolactate		Diacetyl/(diacetyl+acetoin)		Citrate consumed[3]	
	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$
	(m*M*)						(%)		(m*M*)	
CNRZ 212	0.034	0.060	1.35	0.46	<0.05	<0.05	2.4	11.5	5.08	1.31
212M1	0.034	0.076	0.75*	0.26	0.22*	0.35*	4.3*	23.0*	5.53	<0.20
212M2	0.039	0.045	0.73*	0.15*	0.18*	0.14*	5.1*	22.8*	3.21	<0.20
F414	0.011	0.076	0.46	0.84	<0.05	<0.05	2.4	8.3	4.51	1.98
414M1	0.018	0.050	0.33	0.22*	0.16*	0.22*	5.1*	18.5*	4.65	3.20
414M2	0.020	0.081	0.32	0.44*	0.39*	0.44*	5.7*	15.7*	5.00	3.05
414M3	0.017	0.120	0.43	0.66	0.29*	0.67*	3.8*	15.5*	6.05	3.83
414M4	0.021	0.063	0.42	0.28*	0.22*	0.42*	4.8*	18.4*	5.67	2.29
414M5	0.010	0.070	0.14*	0.30*	0.11*	0.11*	6.7*	19.2*	4.39	1.07
414M6	0.009	0.040*	0.12*	0.18*	0.05*	0.12*	6.5*	18.0*	4.70	2.08
414M7	0.010	0.060	0.18*	0.33*	0.08*	0.24*	5.3*	15.4*	2.72	1.92

[1]Cultures were grown under static ($-O_2$) or under aerobic ($+O_2$) conditions at 37°C.

[2]Values after 24 h of culture are the means of three experiments.

[3]Citrate concentration of milk was 8.1 m*M*.

*Values differed from those of the parent strain grown in the same conditions ($P < 0.05$).

0.7 m*M* of acetoin and 0.2 m*M* of α-acetolactate. Production of α-acetolactate was due to the absence of α-acetolactate decarboxylase activity in these mutants. Because α-acetolactate is unstable, it decarboxylates slowly in the culture medium, explaining acetoin production by the mutants. *Lactobacillus rhamnosus* F414 produced 0.46 m*M* of acetoin but no α-acetolactate. Production of α-acetolactate by the F414 mutants varied between 0.05 and 0.39 m*M*. For the parent strains and their mutants, α-acetolactate is the precursor of diacetyl and acetoin. Assuming that no reduction of diacetyl or acetoin occurred via diacetyl and acetoin reductase activities, the ratio of diacetyl to diacetyl plus acetoin represents the proportion of α-acetolactate that underwent an oxidative decarboxylation. This decarboxylation is spontaneous, but α-acetolactate decarboxylase may lead to the formation of small quantities of diacetyl (14). In the static cultures of parent strains, only 2.4% of α-acetolactate underwent an oxidative decarboxylation, and this percentage was almost twice as high in the mutants. Even so, diacetyl production by the mutants was, in general, similar to or barely higher than that by the parent strains. In addition, none of the strains studied was able to consume all of the citrate during the 24-h incubation.

The ratio of diacetyl to diacetyl plus acetoin increased in aerobic conditions (about four times) and was about twice as high in the mutants than in the parent strains (Table 3), although the mutants produced no more diacetyl than the parent strains. These seemingly contradictory observations may be explained by the fact that a large fraction of α-acetolactate was still present in the mutants after 24 h of culture; subsequent degradation would have further increased the diacetyl levels in the cultures of the mutants. In addition, agitation of the cultures resulted in decreased citrate consumption by all the strains compared with static cultures. Mutants 212M1 and 212M2 did not consume any citrate in aerobic conditions, which implies that they were able to produce some diacetyl, acetoin, and α-acetolactate from lactose. Production of 2,3-butanediol was not detected in either agitated or static cultures.

DISCUSSION

As in the case of lactococci, the present results show that mutants of lactobacilli that are deficient in α-acetolactate decarboxylase can be obtained by screening clones that produce α-acetolactate on agar plates. The α-acetolactate decarboxylase of the two *Lb. rhamnosus* strains used in this work is not activated by branched-chain amino acids and is saturated at a substrate concentration lower than 5 m*M*. The properties of this enzyme are, thus, closer to those observed for *Leuconostoc* sp. than for *L. lactis* (16). Nevertheless, the variability within the genus *Lactobacillus* is considerable, and we cannot rule out that some species undergo an allosteric activation of α-acetolactate decarboxylase by branched-chain amino acids, as observed for *L. lactis* (21).

The 9 *Lb. rhamnosus* mutants selected were studied by their growth in milk. The acidification

activity of the majority of the mutants was lower than that of the parent strains, thereby limiting their potential in a manufacturing process. The absence of α-acetolactate decarboxylase activity was not responsible for the decrease in acidifying activity, but this decrease might have been due to other nonspecific mutations. Thus, a sufficiently large number of mutants must be selected in order to use only those clones for which mutagenesis treatment did not modify their characteristics of growth and acidification.

Under partial anaerobiosis, the attenuation of α-acetolactate decarboxylase activity in the strains resulted in the production of α-acetolactate in milk, but not in a significant increase in diacetyl production. This result can be explained by the limitation of the chemical oxidative decarboxylation of α-acetolactate in the absence of oxygen or when the redox potential of the medium is low.

Lactobacillus rhamnosus mutants lacking α-acetolactate decarboxylase produced no more diacetyl than did the parent strains when the medium was aerated. After 24 h of culture, the best mutant produced 0.12 m*M* of diacetyl, and the parent strain produced 0.08 m*M*. Nevertheless, a large fraction of α-acetolactate had not yet been degraded to diacetyl or acetoin, indicating that the maximal concentrations of diacetyl were not reached in mutant cultures. An increase in the diacetyl concentration would, thus, probably occur during the subsequent storage of the cultures.

CONCLUSIONS

Mutants of lactobacilli that are deficient in α-acetolactate decarboxylase can be selected by screening clones producing α-acetolactate in the presence of pyruvate. Nevertheless, the mutants obtained from the two strains of *Lb. rhamnosus* in this study produced no more diacetyl than did the parent strains. Because the genus *Lactobacillus* is relatively heterogeneous, it would be interesting to select α-acetolactate decarboxylase-negative mutants from other species of lactobacilli. The α-acetolactate decarboxylase-negative mutants of certain strains of lactobacilli may possibly have much higher capacities to produce diacetyl and α-acetolactate than do the mutants studied in the present work. The most interesting strains are those that naturally produce large quantities of acetoin in milk because the production of diacetyl and α-acetolactate by α-acetolactate decarboxylase-negative mutants occurs to the detriment of acetoin. Another application of mutants of lactobacilli producing α-acetolactate could be the production of diacetyl in ripened cheeses to favor the production of aromatic compounds by reaction with amino acids (10).

ACKNOWLEDGMENTS

This work was partly financed by contract AIR3-CT94-2010 of the European Union. We thank G. Yonnet and M. C. Binet for technical assistance.

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