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Isolation and Properties of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 Mutants Producing Diacetyl and Acetoin from Glucose

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Following treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, three mutants of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 that produced diacetyl and acetoin from glucose were isolated. The lactate dehydrogenase activity of these mutants was strongly attenuated, and the mutants produced less lactate than the parental strain. The kinetic properties of lactate dehydrogenase of strain CNRZ 483 and the mutants revealed differences in the affinity of the enzyme for pyruvate, NADH, and fructose-1,6-diphosphate. When cultured aerobically, strain CNRZ 483 transformed 2.3% of glucose to acetoin and produced no diacetyl or 2,3-butanediol. Under the same conditions, mutants 483L1, 483L2, and 483L3 transformed 42.0, 78.9, and 75.8%, respectively, of glucose to C₄ compounds (diacetyl, acetoin, and 2,3-butanediol). Anaerobically, strain CNRZ 483 produced no C₄ compounds, while mutants 483L1, 483L2, and 483L3 transformed 2.0, 37.0, and 25.8% of glucose to acetoin and 2,3-butanediol. In contrast to the parental strain, the NADH balance showed that the mutants regenerated most of the NAD via NADH oxidase under aerobic conditions and by ethanol production under anaerobic conditions.

Diacetyl is a major flavor compound in many cultured dairy products. This compound is synthesized primarily by leuconostocs and some lactococci that can metabolize citrate. Once a subject of controversy, the biosynthetic pathway of diacetyl is now well known. Citrate is transported inside cells by a citrate permease (22) and is then cleaved to acetate and oxaloacetate by citrate lyase. Oxaloacetate is decarboxylated by oxaloacetate decarboxylase, yielding pyruvate. α -Acetolactate synthase transforms pyruvate to acetaldehyde-thiamine pyrophosphate and condenses it with a second molecule of pyruvate to form α -acetolactate. Diacetyl originates from the chemical oxidative decarboxylation of α -acetolactate (15, 24, 43) and acetoin originates from the decarboxylation of α -acetolactate by α -acetolactate decarboxylase, by reduction of diacetyl by diacetyl reductase, or by nonoxidative chemical decarboxylation (23).

Since lactic acid bacteria produce relatively small quantities of diacetyl (generally less than 5 mg/liter), considerable work has been devoted to increasing its production. Factors such as pH, temperature (4, 32), citrate concentration (28, 33), and oxygen (3) have been studied for their effects on diacetyl production. Metabolic engineering strategies have also been proposed to obtain strains producing larger quantities of diacetyl. These strategies have aimed at overproduction or, on the contrary, at inactivating certain enzymes. McKay and Baldwin (29) isolated a mutant of *Lactococcus lactis* C2 which, in contrast to the parental strain, had very low lactate dehydrogenase activity. This enzymatic defect caused the mutant to produce diacetyl and acetoin at the expense of lactate. Kuila and Ranganathan (27) exposed *L. lactis* subsp. *lactis* biovar diacetylactis S₁ to UV radiation and obtained mutants that produced a mean of 4.5 times more diacetyl and acetoin than the parental strain did. The mean lactate dehydrogenase activity of these

mutants was five times lower than that of the parental strain. In addition, Platteeuw et al. (36) and Gasson et al. (18) reported the inactivation of *L. lactis* lactate dehydrogenase by genetic engineering. The overexpression of α -acetolactate synthase in *L. lactis* also leads to increased production of acetoin (36). Godon et al. (20) studied *ilvBN* genes encoding for an anabolic α -acetolactate synthase involved in the synthesis of branched-chain amino acids (leucine, isoleucine, and valine). This enzyme has a higher affinity for pyruvate than catabolic α -acetolactate synthase does (5). The overexpression of *ilvBN* genes in *L. lactis* leads to diacetyl production that can reach 0.1 mM in citrate-free medium. Several research groups have isolated an α -acetolactate decarboxylase-negative strain from the high diacetyl-producing starter NIZO 4/25 (26, 38) or from Christian Hansen's D1 starter (30). In contrast to other *L. lactis* subsp. *lactis* biovar diacetylactis strains, this one accumulated α -acetolactate in the culture medium. In the presence of oxygen, unstable α -acetolactate undergoes an oxidative decarboxylation, favoring the production of diacetyl at the expense of acetoin (24, 30). Goupil et al. (21) proposed a method for selecting spontaneous α -acetolactate decarboxylase mutants that was applicable to strains of *L. lactis* that were prototrophic for isoleucine and valine. α -Acetolactate decarboxylase mutants were also obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (31). Finally, the inactivation of the gene coding for α -acetolactate decarboxylase by genetic engineering has been reported by Swindell et al. (39).

In light of the results described above, it is possible to employ different strategies to modify metabolic fluxes to obtain the overproduction of diacetyl. Recent work (7) reported the effect of adding citrate to *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 grown in a whey-based medium. The bioconversion yield of citrate to diacetyl and acetoin decreased considerably as the citrate concentration was increased. The authors thus envisaged the possibility of selecting lactococci

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that could produce diacetyl and acetoin from sugars in the absence of citrate. The present work describes a method for selecting this type of mutant, followed by characterization in terms of glucose metabolism in the presence and absence of oxygen. The kinetics of lactate dehydrogenase, the enzyme responsible for the substantial shunt of metabolic flux in these mutants, were also studied.

MATERIALS AND METHODS

Strain. *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 was obtained from the lactic acid bacteria collection of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France). Stock cultures were obtained after incubating the strain for 15 h at 30°C in MRS medium (16) and inoculating 1.5 ml of the culture in 10 ml of MRS containing 15% (vol/vol) glycerol. They were stored without previous incubation at -80°C.

Obtaining mutants. Strain CNRZ 483 was grown in 5 ml of MRS broth. At the end of logarithmic growth, the culture was centrifuged (2,500 × g, 10 min) and washed twice with 100 mM potassium phosphate buffer (pH 7). The bacteria were then resuspended in 1 ml of a solution of NTG (Aldrich, St. Quentin Fallavier, France) at a final mutagen concentration of 50 µg/ml. At this concentration, the mortality rate caused by NTG was close to 90%. After 1 h of incubation at 30°C, the bacteria were washed in buffer and incubated for 2 h at 30°C in MRS broth. They were then homogenized aseptically with an Ultra-Turrax apparatus (IKA, Labortechnik, Stafen, Germany) for 2 min at 20,000 rpm to break the chains. Two hundred 14-mm-diameter petri dishes were inoculated to obtain about 150 colonies per plate. The agar medium used was a derivative of M17 (41), composed of agar (15 g/liter), tryptone (2.5 g/liter), peptic digest of meat (2.5 g/liter), papain digest of soybeans (5 g/liter), sodium glycerophosphate (19 g/liter), L-ascorbic acid (0.5 g/liter), yeast extract (2.5 g/liter), and magnesium sulfate (0.25 g/liter). The pH of the medium was adjusted to 7 with hydrochloric acid, and glucose (5 g/liter) was added aseptically after autoclaving the agar medium at 110°C for 15 min.

After 72 h of incubation at 30°C, replica plating on felt was carried out because the screening used was lethal for the bacteria. The screening system involved detecting clones that could produce diacetyl and acetoin from glucose in the absence of citrate. These compounds were detected directly on the plates by use of a procedure adapted from Phalip et al. (35). A solution containing 50 g of agar (OSI, Maurepas, France) per liter and 5 g of creatine (Sigma, St. Quentin Fallavier, France) per liter was boiled, cooled to 60°C, and then mixed in a proportion of 3:1 (vol/vol) with a freshly prepared solution of α -naphthol (Sigma) at 75 g/l in 2.5 M sodium hydroxide. Screening was done under a ventilated hood by spreading a 20-ml overlay uniformly on the surface of each petri dish. After the agar solidified (30 min), the plates were examined on a light table and clones forming a red halo were recovered from the replicas.

Identification of mutants. Putative mutants were identified to verify that they were not contaminants. API 50 CH strips (BioMérieux, Marcy l'Etoile, France) and the randomly amplified polymorphic DNA (RAPD) method (45, 47), adapted by Tailliez et al. (40), were used for this. Three primers were used, namely, B06 ($5'$ TGCTCTGCCC $3'$), B07 ($5'$ GGTGACGCAG $3'$), and B10 ($5'$ CTGCTGGGAC $3'$).

Culture medium and conditions. To characterize the mutants obtained, cultures were grown in the absence and presence of oxygen. The medium used was identical to that used for screening except for the absence of agar. Anaerobic cultures were prepared in 1-liter conical flasks containing 1 liter of medium inoculated at 3% (vol/vol). Conditions of anaerobiosis within the flasks were maintained by permanently bubbling with nitrogen (250 ml/h). Aerated cultures were grown in 2-liter conical flasks containing 500 ml of medium inoculated at 3% (vol/vol). The flasks were incubated on a shaker at 200 rpm. All cultures were incubated at 30°C.

Analysis of biomass, products, and metabolites. The absorbance of cultures at 575 nm was measured with a spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). Cultures with an A_{575} higher than 0.6 were diluted in sterile medium. Dry weight (in grams per liter) was calculated from the A_{575} by using the correlation of 1 A_{575} unit equal to 0.51 ± 0.02 g (dry weight). Diacetyl and acetoin were assayed by the colorimetric method of Walsh and Cogan (44). α -Acetolactate was assayed by the colorimetric method described by Monnet et al. (30). Glucose, L-lactate, acetate, pyruvate, ethanol, acetaldehyde, and formate were assayed with Boehringer (Mannheim, Germany) kits. The concentrations of 2,3-butanediol were determined by high-performance liquid chromatography with the method described by Bassit et al. (3).

Assays of enzymatic activities. At the end of growth, culture media were centrifuged at $14,000 \times g$ for 15 min at +4°C. After washing in 50 mM sodium phosphate buffer (pH 7), the bacteria were resuspended (1:20, vol/vol) in the same buffer and treated five times in a Branson (Carouge-Geneva, Switzerland) sonifier for 1 min at 0°C and 50 W. After another centrifugation ($14,000 \times g$ for 30 min), enzymatic activities were determined in the supernatant. Lactate dehydrogenase was measured by monitoring the decrease in A_{340} (11). The assay was performed in 50 mM Tris-maleate buffer (pH 7) containing 100 µl of cell extract, 10 mM sodium pyruvate, 1 mM fructose-1,6-diphosphate, and 0.15 mM NADH

(42). Acetoin and diacetyl reductase were measured as described by Cogan (11). These enzyme activities were corrected for NADH oxidase activities, determined under the same conditions, but in the absence of substrate (pyruvate, diacetyl, and acetoin for lactate dehydrogenase, diacetyl reductase, and acetoin reductase, respectively). NADH oxidase activities given below (see Table 4) were measured with 0.13 mM NADH as described by Anders et al. (2). One enzyme unit was defined as 1 µmol of NADH oxidized min^{-1} . α -Acetolactate synthase activity was determined by measuring acetoin production from pyruvate (11). The reaction mixture contained 100 µl of cell extract, 80 mM sodium pyruvate, and 0.21 mM thiamine pyrophosphate in 100 mM sodium phosphate buffer (pH 6.5). After 15 min of incubation at 45°C, 200 µl of 0.5 M HCl was added to stop the reaction and to convert α -acetolactate to acetoin. The acetoin formed was then measured by the colorimetric method of Westerfeld (46). α -Acetolactate decarboxylase activity was measured as described previously (34) under optimal conditions with 50 mM D- α -acetolactate and in the presence of 40 mM L-leucine. One unit of α -acetolactate synthase (α -acetolactate decarboxylase) activity was defined as the formation of 1 µmol of α -acetolactate (acetoin) per min. Protein concentrations were determined by the method of Bradford (8), with bovine serum albumin as standard protein.

Lactate dehydrogenase kinetics. The effect of pyruvate, NADH, and fructose-1,6-diphosphate concentrations on the activity of lactate dehydrogenase from strain CNRZ 483 and its mutants was determined. The apparent K_m was defined as that substrate concentration at which velocity was half of the maximum ($V_{\max}$). Lactate dehydrogenase activity was measured as described above. A special optical glass cell with a 1-mm pathlength (Hellma 100-OS; Polyabo, Strasbourg, France) enabled concentrations of NADH higher than 0.15 mM to be tested.

RESULTS

Obtaining mutants. After the mutagenesis of *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483, 30,000 colonies were screened for potential production of diacetyl and acetoin from glucose. Three positive colonies were detected: one colony formed a weak red halo (483L1), and the halos of the other two colonies were intense (483L2 and 483L3). The biochemical characters of strain CNRZ 483 and of the three mutants (483L1, 483L2, and 483L3) were identical, as determined with API 50 CH strips. Similarly, the RAPD profiles of the parental strain and the mutants were similar, with a percentage of similarity higher than 94% (results not shown). Thus, clones 483L1, 483L2, and 483L3 had the same origin as strain CNRZ 483.

Growth and production of metabolites by strain CNRZ 483 and the three mutants. Batch cultures in the presence and absence of oxygen were prepared to characterize the parent strain and mutants 483L1, 483L2, and 483L3. Bacterial growth and acidifying activity were monitored, and the principal metabolites of glucose metabolism were assayed (Fig. 1).

(i) Bacterial growth and acidification. Under anaerobic conditions, the final dry weight of strain CNRZ 483 and mutant 483L1, a reflection of total bacterial concentration, was close to 0.90 g/liter (Table 1) and was higher (about 30%) for mutants 483L2 and 483L3. The growth rate of strain CNRZ 483 (0.82 h^{-1}) was slightly higher than those of the three mutants, and the same trends were observed under aerobic conditions. In addition, the maximum growth rates of strains 483, 483L2, and 483L3 were lower under aerobic than anaerobic conditions.

The final pH of the medium after anaerobic growth of strain CNRZ 483 was 5.06, lower than that of mutants 483L1, 483L2, and 483L3 (Table 1). This effect was accentuated under aerobic conditions since the final pH increased for all the strains, in particular for mutants 483L2 and 483L3. This weak acidifying power could explain the higher final biomass. There is probably a lower degree of acid inhibition in these mutants.

(ii) Accumulation of metabolic products. Regardless of culture conditions, glucose ($\sim 26 \text{ mM}$) was totally consumed by all strains (Table 2). The three mutants produced less lactate than strain CNRZ 483 did anaerobically, in particular mutants 483L2 and 483L3, which produced 80% less than the parental strain. Higher ethanol production compensated for the de-

FIG. 1. Metabolic pathway showing the principal routes of glucose utilization by lactococci. 1, triose-phosphate dehydrogenase; 2, lactate dehydrogenase; 3, pyruvate formate lyase; 4, pyruvate dehydrogenase; 5, α -acetolactate synthase; 6, α -acetolactate decarboxylase; 7, diacetyl reductase; 8, acetoin reductase; 9, oxidative decarboxylation of α -acetolactate; 10, acetate kinase; 11, aldehyde dehydrogenase; 12, alcohol dehydrogenase. CoA, coenzyme A.

creased quantities of lactate produced by the three mutants. In contrast to the parental strain that produced very little ethanol (0.7 mM), mutants 483L1, 483L2, and 483L3 produced 4.6, 20.0, and 19.4 mM, respectively. In parallel, the quantity of formate produced by the mutants was also very high, in particular for 483L2 and 483L3. In the presence of oxygen, lactate production by all strains decreased. The three mutants also produced less ethanol and formate, while the parental strain produced none at all. Considerable production of diacetyl and acetoin from glucose compensated for the decreased production of ethanol and lactate by the mutants. Strain CNRZ 483 could not produce diacetyl from glucose, whereas the three mutants 483L1, 483L2, and 483L3 produced the compound (0.2 to 0.3 mM). In parallel, the three mutants produced 10.5, 19.8, and 19.2 mM of acetoin, respectively, while the parental strain produced only traces (0.6 mM). Under anaerobic conditions, only mutants 483L2 and 483L3 still produced acetoin, at concentrations two to four times lower than that produced in the presence of oxygen. 2,3-Butanediol production was observed only for mutants 483L1, 483L2, and 483L3 grown anaerobically and for 483L2 and 483L3 grown in the presence of oxygen. The bioconversion yield of glucose to diacetyl, acetoin, and 2,3-butanediol (RB) was calculated as follows: RB = (diacetyl + acetoin + 2,3-butanediol)/glucose. This yield in strain CNRZ 483 was limited to 2.3% under aerobic conditions and increased considerably in mutants 483L2 and 483L3, reaching

78.9 and 75.8%, respectively. In the presence or absence of oxygen, all strains produced only small quantities of acetaldehyde (maximum, 0.4 mM) and of pyruvate (maximum, 0.5 mM). In parallel, no strain accumulated α -acetolactate. As was observed by Cogan et al. (12), strain CNRZ 483 produced more acetate in the presence of oxygen, which was not the case for the mutants that produced less acetate under aerobiosis.

TABLE 1. Growth and acidification of *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 and mutants^a

Strain	O ₂	Biomass (g/liter)	$\mu_{\max}$ (h ⁻¹) ^b	Final pH
CNRZ 483	—	0.89	0.82	5.06
	+	1.02	0.71	5.45
483L1	—	0.92	0.64	5.33
	+	1.02	0.69	6.01
483L2	—	1.18	0.78	5.56
	+	1.19	0.59	6.46
483L3	—	1.19	0.66	5.46
	+	1.23	0.59	6.44

^a Strains were cultivated at 30°C in modified M17 broth in the presence (+) or absence (—) of oxygen.

^b The maximum growth rate ($\mu_{\max}$) was determined by the slope of a plot of ln absorbance versus time.

TABLE 2. Glucose consumed and products formed by *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 and mutants^a

Measurement (unit)	CNRZ 483		483L1		483L2		483L3	
	–O ₂	+O ₂	–O ₂	+O ₂	–O ₂	+O ₂	–O ₂	+O ₂
Glucose consumed (mM)	26.0	26.5	25.9	25.5	26.0	26.6	26.0	26.0
L-Lactate (mM)	50.3	45.7	39.2	24.3	6.9	4.5	8.5	2.6
Ethanol (mM)	0.7	ND ^b	4.6	3.3	20.0	3.9	19.4	1.6
Formate (mM)	2.4	ND	4.3	ND	27.0	0.5	24.5	1.7
Diacetyl (mM)	ND	ND	ND	0.2	ND	0.3	ND	0.2
Acetoin (mM)	ND	0.6	ND	10.5	8.3	19.8	5.1	19.2
2,3-Butanediol (mM)	ND	ND	0.5	ND	1.3	0.9	1.6	0.3
Acetaldehyde (mM)	0.1	0.4	0.3	0.1	0.2	0.1	0.2	0.1
Pyruvate (mM)	0.5	0.5	0.2	0.2	0.1	0.4	0.4	0.4
Acetate (mM)	3.0	5.5	4.7	3.2	6.6	3.8	8.3	5.3
α-Acetolactate (mM)	ND	ND	ND	ND	ND	ND	ND	ND
Theoretical CO ₂ (mM) ^c	1.4	7.1	6.3	28.0	19.0	49.3	16.8	44.7
Carbon balance (%) ^d	105.0	100.6	96.5	103.0	101.9	102.8	96.5	95.0

^a Strains were cultivated at 30°C in modified M17 broth in the presence or absence of oxygen (+O₂ and –O₂, respectively). The values are means of at least three measurements. The standard error was lower than 5% for all compounds except 2,3-butanediol, where it was lower than 15%.

^b ND, not detectable.

^c Theoretical CO₂ = 2 × (diacetyl + acetoin + 2,3-butanediol) + (acetate + ethanol + acetaldehyde – formate).

^d Carbon balance calculated by taking into account theoretical CO₂.

The theoretical production of CO₂ by the different strains was calculated from the metabolic pathway (Fig. 1). Anaerobically, the mutants produced more CO₂ than strain CNRZ 483 did (Table 2). All strains produced more CO₂ under aerobic conditions, in particular mutants 483L2 and 483L3, which produced 49.3 and 44.7 mM CO₂, respectively. The carbon balance was calculated from the different fermentation products arising from glucose metabolism. Recovered carbon varied from 95.0 to 105.0% as a function of the strains and culture conditions.

(iii) **NADH balance.** The quantities of NADH produced and consumed were calculated on the basis of the final concentrations of products of glucose metabolism (Table 3). Anaerobically, strains CNRZ 483 and 483L1 reoxidized 94.2 and 68.7%, respectively, of the NADH produced by the lactate dehydrogenase-catalyzed formation of lactate. These strains also used ethanol and acetaldehyde biosynthetic pathways to regenerate NAD. Strains CNRZ 483 and mutant 483L1 reoxidized small quantities of NADH by ethanol formation (2.6 and 16.1%, respectively). This mutant also reoxidized 0.9% of NADH by the formation of 2,3-butanediol. The levels of NADH reoxidized by lactate dehydrogenase in mutants 483L2 and 483L3 were limited to 13.3 and 15.3%, respectively. On the other hand, more than 70% of the reduced coenzyme was reoxidized

by ethanol formation. In addition, these mutants reoxidized a mean of 2.7% of the NADH resulting from 2,3-butanediol synthesis. The difference between NADH produced and consumed was between 3.0 and 13.8% of total NADH produced as a function of the strain. This excess could have resulted from the imprecision of certain assays and/or the existence of NADH reoxidation pathways not taken into account by our calculations. It cannot be attributed to NADH oxidase, since the cultures were grown in the absence of oxygen.

Under aerobic conditions, all of the strains reoxidized less NADH through the formation of lactate, ethanol, and acetaldehyde than under anaerobic conditions. Mutants 483L2 and 483L3 reoxidized 13.0 and 5.6%, respectively, of the NADH produced by ethanol synthesis. The difference between the quantity of NADH produced and that consumed could be due to the participation of two enzymes not mentioned in Table 3, i.e., NADH oxidase and diacetyl reductase. Nevertheless, it has been shown that the same enzyme, acetoin reductase, catalyzes the reduction of diacetyl (to acetoin) and of acetoin (to 2,3-butanediol) (13, 19). In a mixture of acetoin and diacetyl, acetoin is the preferred substrate of acetoin reductase (23). There is thus probably less reoxidation of NADH via the transformation of diacetyl to acetoin than reoxidation due to the transformation of acetoin to 2,3-butanediol. Under aerobic

TABLE 3. Comparison of NADH balances in *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 and mutants^a

Strain	O ₂	NADH produced (mM)			NADH consumed (mM)					Difference ^b
		Triose-P-dehydrogenase ^c	Pyruvate dehydrogenase ^c	Total	Lactate dehydrogenase ^c	Aldehyde dehydrogenase ^c	Alcohol reductase ^c	Acetoin dehydrogenase ^c	Total	
CNRZ 483	–	52.0	1.4	53.4	50.3 (94.2) ^d	0.1 (0.2)	1.4 (2.6)	0.0	51.8	1.6 (3.0)
	+	53.0	5.9	58.9	45.7 (77.6)	0.4 (0.7)	0.0	0.0	46.1	12.8 (21.7)
483L1	–	51.8	5.3	57.1	39.2 (68.7)	0.3 (0.5)	9.2 (16.1)	0.5 (0.9)	49.2	7.9 (13.8)
	+	51.0	6.6	57.6	24.3 (42.2)	0.1 (0.2)	6.6 (11.5)	0.0	31.0	26.6 (46.2)
483L2	–	52.0	0.0	52.0	6.9 (13.3)	0.2 (0.4)	40.0 (77.0)	1.3 (2.5)	48.4	3.6 (6.9)
	+	53.2	7.3	60.5	4.5 (7.4)	0.1 (0.2)	7.8 (13.0)	0.9 (1.5)	13.3	45.2 (74.7)
483L3	–	52.0	3.4	55.4	8.5 (15.3)	0.2 (0.4)	38.8 (70.0)	1.6 (2.9)	49.1	6.3 (11.4)
	+	52.0	5.3	57.3	2.6 (4.5)	0.1 (0.2)	3.2 (5.6)	0.3 (0.5)	6.2	51.1 (89.2)

^a Mutants were cultivated at 30°C, in modified M17 broth, in the presence (+) or absence (–) of oxygen (O₂).

^b The difference between NADH produced and NADH consumed.

^c Enzyme involved in glucose utilization.

^d Values in parentheses are percentages of NADH consumed via the different pathways related to NADH produced.

TABLE 4. Enzymatic activities measured in cell extracts of *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 and mutants^a

Enzyme	Sp act (U/mg of protein) ^b							
	CNRZ 483		483L1		483L2		483L3	
	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂	-O ₂	+O ₂
Lactate dehydrogenase	30.76	23.08	2.80	2.34	0.09	0.08	0.09	0.09
NADH oxidase	0.02	0.17	0.03	0.29	0.08	0.30	0.07	0.41
α -Acetolactate synthase	0.18	0.65	0.17	0.50	0.26	0.49	0.16	0.64
α -Acetolactate decarboxylase	0.23	0.49	0.19	0.34	0.29	0.35	0.18	0.45
Acetoin reductase	<0.02	<0.02	0.03	0.05	0.04	0.04	0.05	0.05
Diacetyl reductase	0.09	0.09	0.18	0.25	0.15	0.15	0.31	0.19

^a The strains were grown for 8 h at 30°C in modified M17 broth in the presence or absence of oxygen.

^b The values are the means of at least three measurements. The standard error was lower than 10% for all enzymes except α -acetolactate decarboxylase, where it was lower than 20%.

conditions, however, the latter pathway reoxidizes only a very small quantity of NADH (Table 3). It is thus probable that the difference between NADH produced and consumed essentially represents the quantity of NADH reoxidized by NADH oxidase. This enzyme would reoxidize 21.7% of the NADH produced by strain CNRZ 483, a percentage that increased considerably in mutants 483L1, 483L2, and 483L3 (46.2, 74.7, and 89.2%, respectively).

Enzymatic activities. Some enzymatic activities were assayed at the end of culture to explain the modifications of glucose metabolism in the mutants (Table 4). The results show that under anaerobiosis, the specific activity of lactate dehydrogenase in strain CNRZ 483 was 30.76 U/mg, higher than that observed under aerobiosis (23.08 U/mg). In the presence or absence of oxygen, this activity decreased considerably in the other strains, in particular mutants 483L2 and 483L3: lactate dehydrogenase activity in this case was less than 0.4% that of the parental strain. The specific activity of NADH oxidase was higher in the presence of oxygen, especially in mutants 483L2 and 483L3 (0.30 and 0.41 U/mg, respectively). Similarly, the activities of α -acetolactate synthase and α -acetolactate decarboxylase were higher in the presence of oxygen in all strains, but no major difference in α -acetolactate synthase and α -acetolactate decarboxylase activities was noted among the different strains. In the presence or absence of oxygen, acetoin reductase activity in strain CNRZ 483 was lower than 0.02 U/mg. This activity was higher in the mutants and was between 0.03 and 0.05 U/mg. Diacetyl reductase activity in strain CNRZ 483 (0.09 U/mg) was also lower than that of the three mutants, where activities were between 0.15 and 0.31 U/mg.

Kinetic parameters of lactate dehydrogenase. Lactate dehydrogenase activity in mutants 483L2 and 483L3 was approximately 300 times lower than that in the parental strain. It was nevertheless surprising to note that the corresponding lactate production was only 6.5 to 17.6 times lower, depending on the presence or absence of oxygen. This explains why we investigated the saturation of lactate dehydrogenase by pyruvate, NADH, and fructose-1,6-diphosphate in the different strains (Fig. 2). The kinetic constants (K_m and V_{max}) of lactate dehydrogenase of the different strains are shown in Table 5. The saturation curve of strain CNRZ 483 lactate dehydrogenase by pyruvate was hyperbolic (Fig. 2A). The apparent K_m of lactate dehydrogenase in strain 483L1 was close to that of the parental strain, but the V_{max} was about four times lower. The V_{max} value of the lactate dehydrogenases of mutants 483L2 and 483L3 was lower than that of the parental strain. The affinity of mutant 483L2 lactate dehydrogenase for pyruvate was very low ($K_m = 29.0$ mM), and the saturation curve was sigmoid. When lactate dehydrogenase was saturated by NADH (Fig. 2B), the appar-

FIG. 2. Saturation of lactate dehydrogenase by pyruvate in the presence of 3 mM NADH and 10 mM fructose-1,6-diphosphate (A), by NADH in the presence of 100 mM pyruvate and 10 mM fructose-1,6-diphosphate (B), and by fructose-1,6-diphosphate in the presence of 3 mM NADH and 100 mM pyruvate (C). Assays were conducted in cell extracts obtained after 8 h of growth at 30°C in modified M17 broth. Symbols: $\blacklozenge$, *L. lactis* subsp. *lactis* biovar diacetylactis CNRZ 483; $\square$, mutant 483L1; $\circ$, mutant 483L2; $\triangle$, mutant 483L3.

TABLE 5. Kinetic constants of lactate dehydrogenase in strain CNRZ 483 and mutants^a

Substrate	CNRZ 483		483L1		483L2		483L3	
	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)
Pyruvate	2.6	40	3.0	10.6	29.0	21.3	1.0	9.0
NADH	0.07	35.8	0.17	12.0	1.90	27.5	2.90	16.0
Fructose-1,6-diphosphate	0.03	36.0	0.14	10.0	0.89	18.5	1.10	9.0

^a Assays were conducted with cell extracts obtained after 8 h of culture at 30°C in modified M17 broth.

ent K_m and V_{max} of the CNRZ 483 enzyme were 0.07 mM and 35.8 U/mg, respectively. The lactate dehydrogenases of mutants 483L1, 483L2, and 483L3 had higher apparent K_m values than the parental strain and lower V_{max} values (Table 5). There were also differences in lactate dehydrogenase saturation by fructose-1,6-diphosphate (Fig. 2C). The lactate dehydrogenase saturation curves of all the strains were sigmoid. The apparent K_m values in the three mutants were higher than in the parental strain, while V_{max} values were lower.

DISCUSSION

This work describes a method for isolating mutants of lactococci that can produce diacetyl and acetoin from glucose in the absence of citrate. This metabolic shunt is due primarily to the change of an enzyme, lactate dehydrogenase. The kinetic study of lactate dehydrogenases of the different mutants has shown that treatment with the mutagen did not totally inactivate the enzyme but rather modified its properties. As in the case of all lactococci, the lactate dehydrogenases of the mutants and the parental strain were active only in the presence of fructose-1,6-diphosphate (9, 14, 17, 25). When the enzyme was saturated by pyruvate, the apparent K_m of strain CNRZ 483 lactate dehydrogenase (2.6 mM) was higher than that reported by Crow and Pritchard (1.5 mM) (14). The K_m and V_{max} values of the mutants differed from those of the parental strain. In mutant 483L2, the K_m was very high, reflecting a low affinity for pyruvate. The apparent K_m of CNRZ 483 lactate dehydrogenase for NADH was the same as that reported by Crow and Pritchard (0.08 mM) (14). Nevertheless, the affinity of the mutant lactate dehydrogenases for NADH was lower, particularly in mutant 483L3, whose apparent K_m was about 40 times higher than that of the parental strain. Regardless of the strain, the curve of lactate dehydrogenase saturation by fructose-1,6-diphosphate was sigmoid, suggesting a positive cooperativity in fructose-1,6-diphosphate binding. Enzyme affinity for fructose-1,6-diphosphate was lower in the mutants, in particular mutant 483L3, whose apparent K_m was about 40 times higher than that of the parental strain.

Under anaerobic conditions, the mutants produced less lactate than the parental strain did, probably because of their lower lactate dehydrogenase activity. In contrast to the parental strain, which mainly used lactate dehydrogenase to regenerate NAD, the mutants reoxidized a portion of the coenzyme for ethanol synthesis, thus producing large quantities of formate. In comparison to the parental strain, the quantity of ethanol produced was approximately half of the lactate deficit (lactate produced by the parental strain minus lactate produced by each mutant), since 2 mol of NAD was regenerated per mol of ethanol produced. Mutants 483L2 and 483L3 reoxidized more than 70% of the NADH by this pathway. These mutants also regenerated a small quantity of NAD by the formation of 2,3-butanediol. Formate and ethanol production by lactate dehydrogenase-negative mutants had been reported

by Platteuw et al. (36). Pyruvate is considered to be toxic for the cell, explaining why pyruvate not used to regenerate NAD was shunted towards the synthesis of other products such as acetate and acetoin in the mutants. As was observed for strain CNRZ 483, mutant 483L1 produced no acetoin. Attenuation of lactate dehydrogenase in this mutant was probably not sufficient to yield the synthesis of large quantities of acetoin under anaerobiosis. In addition, no strain did produce diacetyl under anaerobic conditions. It is currently accepted that diacetyl formation from α -acetolactate occurs only in the presence of oxygen or under oxidizing conditions (15, 43).

All strains produced less lactate under aerobic conditions than in the absence of oxygen, which probably resulted from the reduced activity of lactate dehydrogenase in the presence of oxygen. A recent study has shown that lactate dehydrogenase activity in strain CNRZ 483 decreased as the oxygen concentration increased (6). Smart and Thomas (37) also showed that in the presence of oxygen, the intracellular concentration of fructose-1,6-diphosphate was lower, thereby resulting in decreased lactate dehydrogenase activity *in vivo*. In the presence of oxygen, a portion of the NAD is regenerated via NADH oxidase. As observed by Bruhn and Collins (10) and by Bassit et al. (3), this enzyme activity increases in the presence of oxygen. Strain CNRZ 483 reoxidized 21.7% of NADH with this enzyme, and the percentage was higher in the mutants (up to 89.2% in strain 483L3). The quantities of ethanol and formate produced decreased in the presence of oxygen, probably because of the oxygen sensitivity of pyruvate formate lyase (1). Larger quantities of diacetyl and acetoin were produced in the presence of oxygen. Since lactate and ethanol production were lower, a larger quantity of pyruvate was available for the synthesis of diacetyl and acetoin. Furthermore, as observed by Cogan et al. (12) and Bassit et al. (3), α -acetolactate synthase activity in all strains increased under aerobiosis, thus favoring acetoin synthesis. In mutants 483L2 and 483L3, almost 80% of glucose was transformed to diacetyl, acetoin, and 2,3-butanediol. It was also shown that the activity of α -acetolactate decarboxylase increased in all the strains in the presence of oxygen. All lactate dehydrogenase-attenuated mutants produced much more acetoin than diacetyl, probably as a result of their α -acetolactate decarboxylase activity. This is why this type of mutant cannot be selected by use of a diacetyl-specific colorimetric reaction on petri dishes, such as that described by Monnet et al. (31). The screening method used in the present work leads to the simultaneous detection of the production of diacetyl and, above all, acetoin, which predominates in lactate dehydrogenase-attenuated mutants.

In summary, this work has led to the isolation and characterization of *L. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 mutants that produced diacetyl and acetoin from glucose in the absence of citrate. The mutations caused a severe attenuation of lactate dehydrogenase activity. Genetic engineering inactivation of the gene coding for lactate dehydrogenase has been described by Platteuw et al. (36) and Gasson et al. (18).

The principal difference between our work and that of these authors is that we did not select mutants lacking lactate dehydrogenase activity but rather those exhibiting various levels of attenuation of this enzyme activity. This will enable mutants with a desired equilibrium between acidifying activity and aroma production to be selected. In addition, the kinetic properties of lactate dehydrogenase from mutants, i.e., affinity for pyruvate, NADH, and fructose-1,6-diphosphate, were different from those of the parental strain. This is probably due to a structural modification on the enzyme in the mutants. To confirm this hypothesis, it will be necessary to study the sequences of the gene coding for lactate dehydrogenase in the mutants and the parental strain. In addition, the comparison between gene sequences and the corresponding kinetic properties of lactate dehydrogenase should enable us to better understand the structure-function relationship of this enzyme.

The application of these mutants in the production of dairy products is being studied. A complementary approach would involve selecting α -acetolactate decarboxylase-negative mutants from these strains. Theoretically, these double mutants would produce α -acetolactate and diacetyl at the expense of acetoin.

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