**A method for the selection of *Lactococcus lactis* mutants producing excess
carbon dioxide**

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One of the characteristics of Roquefort cheese is the presence of irregularly shaped openings. Although many factors affect the development of opening in blue-veined cheeses (Martley & Crow, 1996), the limiting step in the case of Roquefort cheese is the production of carbon dioxide by lactic acid bacteria (J.-P. Reverbel, personal communication). Most of the opening occurs after moulding, the process is difficult to control and many manufacturing runs result in cheeses with an insufficient opening. Concentrated suspensions of *Leuconostoc* strains are used to increase the production of carbon dioxide (Devoyod & Muller, 1969), but it would clearly be of use to have microorganisms that produce larger quantities of the gas. McKay & Baldwin (1973) isolated a spontaneous mutant of *Lactococcus lactis* that produced more acetoin and carbon dioxide than the parental strain. This mutant was lactate dehydrogenase (LDH) deficient, that favoured the conversion of pyruvate into end products other than lactate. Following nitrosoguanidine mutagenesis, we isolated three *Lc. lactis* mutants whose LDH activities were reduced to varying extents and which led to variable production of carbon dioxide (Boumerdassi *et al.* 1997). Subsequent work showed that these mutants were unstable on successive subcultures in milk or synthetic broth (unpublished results).

The aim of this work was to select a large number of *Lc. lactis* mutants producing excess carbon dioxide. This would increase the probability of selecting stable mutants and also provide a collection of strains with differing gas production activities. Currently available screening methods, however, are inconsistent with processing large numbers of mutants. This is why we developed an improved screening method.

MATERIALS AND METHODS

Strains used

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* RD311 was obtained from Texel (F-86220 Dangé-Saint-Romain, France) and CNRZ 483 from the lactic acid bacteria collection of the Institut National de la Recherche Agronomique of Jouy-en-Josas (F-78352 Jouy-en-Josas, France). Strains 483L1 and 483L2 are mutants with attenuated LDH activity isolated by Boumerdassi *et al.* (1997) from strain CNRZ 483.

Mutagen treatments

Strain RD311 was grown at 30°C in 10 ml of M17 broth (Terzaghi & Sandine, 1975). Cells were harvested at the end of the exponential phase of growth by centrifugation, washed with 10 ml of 0.1 M Na phosphate buffer, pH 7 and resuspended in 1 ml of buffer. Treatments with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were carried out by mixing the cell suspension with 1 ml of solutions of varying NTG concentrations, and incubating for 1 h at 30°C. The cells were recovered by centrifugation, washed with 10 ml of Na phosphate buffer and resuspended in 10 ml of M17 broth. After incubating for 1 h at 30°C, the culture was diluted 100 times in the buffer and dispersed with a mechanical blender (Ultra-Turrax® model T25; Ika Labortechnik, D-79217 Staufen, Germany) for 1 min. The cells were then inoculated in screening medium to obtain about 1000 colonies per Petri dish. When spontaneous mutants were sought, the same protocol was used, replacing the NTG solution with the buffer. When mutagenesis was carried out with UV irradiation, cells harvested at the end of exponential growth were recovered in 10 ml of Na phosphate buffer. The suspension was placed in a 90 mm diameter Petri dish and exposed to UV radiation at 254 nm (UVSL-58 UV lamp; Ultra-Violet Products Inc., San Gabriel, Cal. 91778, USA). The cells were

centrifuged and resuspended in M17 broth and then treated in the same way as for NTG-treated cells. After mutagenesis, all the mutants were purified at least four times by successive plating on screening medium and selecting individual colonies. The purity and identity of the resulting isolates were then confirmed by several examinations. When cultivated at 30°C, all the isolates had the following properties (except when stated, all cultures on agar plates were incubated for 2 days before examination) : Gram-positive, cocci-shaped and catalase negative microorganisms; blue colonies formed on Kempler & McKay agar (1980); arginine positive on M16-BCP agar (Thomas, 1973); absence of growth on M17 agar supplemented with 65 g/l NaCl; growth on M17 agar supplemented with 20 g/l NaCl; fermentation of lactose, galactose, fructose, maltose and ribose when tested on a modified MRS agar (De Man *et al.* 1960) in which glucose and citrate were omitted, and containing 5 g/l of the sugar substrate and 0.05 g/l of bromocresol purple; absence of growth on MRS agar supplemented with 20 µg/ml vancomycin when incubated for 5 days. All the isolates grew on M17 agar when incubated for 7 days at 10°C or for 2 days at 40°C. None of the isolates grew on M17 agar at 45°C. All these properties were also observed when the parent strain was examined.

Screening medium

Agar media containing 2,3,5-triphenyl tetrazolium (TTC) were tested for their capacity to detect colonies of *Lc. l. l. diacetylactis* CNRZ 483 mutants with attenuated LDH activity. These media were prepared from a basic medium called LDHA, derived from M17 broth: tryptone (2.5 g), papain digest of soybeans (5 g), peptic digest of meat (2.5 g), meat extract (5 g), yeast extract (2.5 g), L-ascorbic acid (0.5 g), magnesium sulphate (0.25 g), K₂HPO₄ (0.5 g), glucose (10 g), and agar (15 g). The ingredients were dissolved in 1 l of distilled water and pH was adjusted to 7 with hydrochloric acid. The medium was autoclaved at 115°C for 15 min, after which its temperature was held at 50°C and 10 ml of a filter-sterilised TTC solution

(10 g/l) were added. The medium was poured into 140 mm diameter Petri dishes which were incubated at 30°C for 48 h after inoculation.

Measurement of CO₂ production

5 Skimmed ewes milk powder was obtained from the Société des Caves (F-12250 Roquefort-sur-Soulzon, France). It contained 407.6 g of lactose and 439.5 g of total protein per kg. Growth in this medium was similar to that in fresh ewes milk, but higher than in skimmed cows milk, probably because of the lower buffering capacity of the latter (J.-P. Reverbel, personal communication). Following a 24 h preculture at 30°C in 5 ml of M17
10 broth, the strains were inoculated at the concentration of 1% in 20 ml of reconstituted skimmed ewes milk (130 g/l) that had been sterilised at 110°C for 15 min. The 60 ml culture vessels with a magnetic stirring bar were then sealed with a rubber septum and an aluminium capsule. After incubating for 24 h at 30°C, gas production was determined as described by Kihal (1995). The method involves measuring the displacement of a column of water in a
15 40 ml graduated burette. The bottom of the burette was immersed in a water tank and the top was closed with a tap linked to a syringe, whose function was to adjust the water level to zero at the start of the measurement. All measurements were done at 20°C. The septum of the vessels was pierced with a needle connected to the top of the burette filled with water and 3 ml of 2 M HCl were added to the vessel with a syringe and needle. The volume of water
20 displaced was measured after stirring the culture for 3 min. CO₂ production by cultures was calculated by considering that one mole of CO₂ is equivalent to a volume of 24.04 l (at 20°C and 1 atm.). It was observed that a portion of the CO₂ remained in the medium in the form of H₂CO₃, because of the presence of CO₂ in the gas phase. In order to assay total CO₂, it was necessary to calibrate the system, e.g. with sodium bicarbonate in milk. The following
25 relationship was obtained by polynomial regression:

$$B = -0.0014 \cdot A^2 + 1.4768 \cdot A \quad (r^2 = 0.999)$$

where A is CO₂ production (in mM) that does not take into account the CO₂ remaining in the medium and B is the corrected production of CO₂.

In practice, it would also be possible to use a linear regression ($B = 1.3035 \cdot A$), but this possibility was not retained here because the value of r^2 was slightly lower (0.995). The coefficients of variation of CO₂ production measurements were generally close to 3%.

RESULTS

Development of a method for screening mutants with attenuated lactate dehydrogenase activity

5 Many microorganisms reduce TTC to formazan, an insoluble compound that colours colonies red or brown, but TTC reduction does not occur in acidic culture media (Lederberg, 1948). Hillman (1978) applied this principle to develop a medium for screening LDH-deficient mutants of *Streptococcus mutans*. We sought conditions for the screening of *Lc. l. l. diacetylactis* CNRZ 483 mutants with attenuated LDH activity by varying the buffering
10 capacity of LDHA medium by supplementing it with different quantities of sodium glycerophosphate. At the concentration of 20 g/l, strain CNRZ 483 formed white colonies. In the same conditions, mutant 483L1 whose LDH activity was 9% that of the parental strain (Boumerdassi *et al.* 1997) formed colonies with a brown centre. Mutant 483L2 whose LDH activity was only 0.3% that of the parental strain formed colonies that were entirely brown. At
15 a concentration of 40 g/l of glycerophosphate, both the parent strain and the mutants formed brown colonies. At lower concentrations of glycerophosphate (0, 5 and 10 g/l) the parent strain and mutant 483L1 formed white colonies, whereas mutant 483L2 formed colonies that were entirely brown or had a brown centre. LDHA medium supplemented with 20 g/l of glycerophosphate, called LDHA-20, was thus used in the remainder of this work to screen for
20 mutants. It enabled the distinction of one colony of mutant 483L1 or 483L2 on a Petri dish among more than 1000 colonies of the parental strain.

Selection of mutants on LDHA-20 agar

 The effect of the UV dose and NTG concentration on the survival of *Lc. l. l. diacetylactis* RD311 was determined (Fig. 1). In the case of both mutagens, the logarithm of
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cell viability decreased linearly with the intensity of mutagen treatment. Four mutagenic treatments were then used to screen for mutants forming brown colonies on LDHA-20 agar (Table 1): incubation of the cells in the absence of NTG (spontaneous mutation), in the presence of 20 µg/ml NTG, in the presence of 200 µg/ml NTG, and after UV irradiation at 44.7 J/m². The highest frequency of brown colonies was obtained with 200 µg/ml of NTG. This frequency decreased by a factor of about two when the NTG concentration was 20 µg/ml, by a factor of about 20 with UV treatment and by a factor of about 200 for spontaneous mutation.

10 *CO₂ production*

After each mutagen treatment, mutants forming brown colonies on LDHA-20 agar were ranked as a function of their CO₂ production (Table 2). For the four mutagen treatments, about half the mutants produced between 15 and 30 mM CO₂, equivalent to the same or slightly higher production as the parental strain that produced 18 mM. In contrast to spontaneous and UV-induced mutants, a portion of the NTG-induced mutants produced less than 15 mM CO₂. Some spontaneous mutants produced more than 90 mM CO₂, whereas some NTG and UV-induced mutants produced more than 105 mM of gas. Compared to the total number of colonies screened (i.e. the number of white colonies plus the number of brown colonies), the percentages of mutants whose CO₂ production was higher than 45 mM were 0.001, 0.109, 0.165 and 0.008% for spontaneous mutation, mutagenesis with 20 µg/ml of NTG, 200 µg/ml of NTG, and UV, respectively.

DISCUSSION

The LDHA-20 medium enabled colonies of parental strain of *Lc. l. l. diacetylactis* CNRZ 483 to be distinguished from those of mutated strains exhibiting attenuated LDH activity. It contains TTC, a redox indicator that can be reduced to formazan by most lactic acid bacteria. It is important to emphasise, however, that TTC is reduced only if the pH is not excessively acid (Lederberg, 1948). Acidifying parental strains thus form white colonies, while mutants with attenuated LDH activity form brown colonies resulting from the formation of a formazan precipitate. Colony colour thus depends on the balance between their acidifying and reducing capacities. In general, formazan precipitates are red. The fact that the precipitates observed on LDHA-20 agar were brown was probably due to the effect of some components present in the medium. Up to now, we have tested 24 different strains of *Lc. l. l. diacetylactis* on LDHA-20 agar. Twenty two of them exhibited the same behaviour as strain CNRZ 483, forming white colonies on LDHA-20 agar, whereas colonies were brown when the glycerophosphate concentration was increased to 40 g/l. Two strains formed brown colonies on LDHA-20, but the colonies were white when the glycerophosphate concentration was between 0 and 10 g/l. For the latter type of strains, it is necessary to reduce the concentration of glycerophosphate in the screening medium to 10 g/l to correctly distinguish wild-type colonies from mutants. It was easier to use LDHA-20 medium to screen for mutants with attenuated LDH activity than the medium described by Boumerdassi *et al.* (1997). LDHA-20 in fact enabled a larger number of colonies per Petri dish to be examined (1000 vs. 150) and did not require treatments of the Petri dishes after the growth of colonies (replica plating on velvet and staining with α -naphthol). It was thus possible to select mutants in a cell suspension containing a very low proportion of mutants, e.g. in the case of screening for spontaneous mutants.

It was observed that about half of the mutants forming brown colonies on LDHA-20 agar produced a similar amount of CO₂ than the parent strain. Several types of mutations may lead to such strains. For example, mutants having the same acidifying capacity as the parental strain, but an increased reducing capacity, should form brown colonies without producing excess CO₂. Also, several mutants forming brown colonies on LDHA-20 agar may have mutations resulting in a poor growth in milk that would decrease their ability to produce large quantities of CO₂. Such mutants would have no technological interest, which is why it is necessary to add a second stage after selecting mutants on LDHA-20 agar, i.e. the measurement of CO₂ production in milk cultures. The procedure described in materials and methods has the advantage of being very precise and simple to use in practice.

The present work centred around the development of a method for the selection of mutants producing excess CO₂. The mutants that were selected will be studied in relation to their metabolism of lactose and citrate in milk, their LDH activity, and their stability.

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FIGURE LEGENDS

Fig. 1. Effect of the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) concentration (●) or the dose of UV radiation (○) on the viability of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* RD311 cells.

Table 1. *Selection of mutants of Lactococcus lactis subsp. lactis biovar. diacetylactis RD311 forming brown colonies on LDHA-20 agar[†]*

Mutagen treatment	Survival rate (%)	Number of colonies examined	Number of brown colonies	Frequency of brown colonies (%)
Incubation in the absence of NTG [‡]	100.0	4.1×10^5	17	4.1×10^{-3}
Incubation with 20 $\mu\text{g/ml}$ of NTG	56.8	3.5×10^4	124	3.5×10^{-1}
Incubation with 200 $\mu\text{g/ml}$ of NTG	9.8	1.7×10^4	121	7.1×10^{-1}
UV irradiation at the dose of 44.7 J/m^2	7.0	3.0×10^5	100	3.3×10^{-2}

5 [†] Defined in text

[‡] Spontaneous mutation; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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Table 2. *CO₂ production by mutants of Lactococcus lactis subsp. lactis biovar. diacetylactis**RD311 forming brown colonies on LDHA-20 agar[†]*

CO ₂ production range (mM)	Number of mutants per class of CO ₂ production (%)			
	Spontaneous mutants	Mutants with 20 µg/ml of NTG [‡]	Mutants with 200 µg/ml of NTG	Mutants with UV irradiation at 44.7 J/m ²
0–15	0.0	4.0	14.0	0.0
15–30	41.2	44.3	44.6	52.0
30–45	35.3	21.0	17.4	23.0
45–60	17.6	8.1	8.3	5.0
60–75	0.0	4.0	5.0	2.0
75–90	0.0	0.8	7.4	8.0
90–105	5.9	7.3	2.5	5.0
105–120	0.0	10.5	0.8	5.0

5 [†] Defined in text[‡] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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