# Method for the selection of *Lactococcus lactis* mutants producing excess carbon dioxide

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(*Received 4 October 1999 and accepted for publication 8 June 2000*)

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 $CO_2$ by lactic acid bacteria (J.-P. Reverbel, pers. comm.). 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 $CO_2$ (Devoyod & Muller, 1969), but it would clearly be useful to have microorganisms that produce larger quantities of the gas. McKay & Baldwin (1974) isolated a spontaneous mutant of *Lactococcus lactis* that produced more acetoin and $CO_2$ than the parent strain. This mutant was lactate dehydrogenase (LDH)-deficient, which 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 produced varying amounts of $CO_2$ (Boumerdassi *et al.* 1997). Subsequent work showed that these mutants were unstable on successive subculture in milk or synthetic broth (El Attar *et al.* 2000).

The aim of our current work was to select a large number of *Lc. lactis* mutants producing excess $CO_2$. 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 are, however, unsuitable for processing large numbers of mutants, which is why we have 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

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https://doi.org/10.1017/S0022029900004490 Published online by Cambridge University Press

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 M17 broth (Terzaghi & Sandine, 1975). Cells were harvested by centrifugation at the end of the exponential phase of growth, washed with 10 ml 0·1 M-sodium phosphate buffer, pH 7 and resuspended in 1 ml 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 at 30 °C for 1 h. The cells were recovered by centrifugation, washed with 10 ml sodium phosphate buffer and resuspended in 10 ml M17 broth. After incubation at 30 °C for 1 h, the culture was diluted 100-fold 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 ~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 u.v. irradiation, cells harvested at the end of exponential growth were recovered in 10 ml sodium phosphate buffer. The suspension was placed in a 90 mm diam. Petri dish and exposed to u.v. radiation at 254 nm (UVSL-58 u.v. lamp; Ultra-Violet Products Inc., San Gabriel, CA 91778, USA). The cells were centrifuged and resuspended in M17 broth and then processed 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 d before examination): Gram-positive, cocci-shaped and catalase-negative microorganisms; blue colonies formed on Kempler & McKay (1980) agar; arginine-positive on M16-BCP agar (Thomas, 1973); absence of growth on M17 agar supplemented with 65 g NaCl/l; growth on M17 agar supplemented with 20 g NaCl/l; 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 sugar substrate/l and 0·05 g bromocresol purple/l; absence of growth on MRS agar supplemented with 20 $\mu$g vancomycin/ml when incubated for 5 d. All the isolates grew on M17 agar when incubated at 10 °C for 7 d or at 40 °C for 2 d. 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. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 mutants with attenuated LDH activity. These media were prepared from a basic medium called LDHA, derived from M17 broth containing 2·5 g tryptone, 5 g papain digest of soyabeans, 2·5 g peptic digest of meat, 5 g meat extract, 2·5 g yeast extract, 0·5 g L-ascorbic acid, 0·25 g $MgSO_4$, 0·5 g $K_2HPO_4$, 10 g glucose and 15 g agar dissolved in 1 l distilled water with pH adjusted to 7 with HCl. The medium was autoclaved at 115 °C for 15 min, after which its temperature was held at 50 °C and 10 ml filter-sterilized TTC solution (10 g/l) was added. The medium was poured into 140 mm diam. Petri dishes which were incubated at 30 °C for 48 h after inoculation.

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*Measurement of carbon dioxide production*

Skim ewes' milk powder was obtained from the Société des Caves (F-12250 Roquefort-sur-Soulzon, France). It contained 407·6 g lactose/kg and 439·5 g total protein/kg. Growth in this medium was similar to that in fresh ewes' milk, but higher than in skim cows' milk, probably because of the lower buffering capacity of the latter (J.-P. Reverbel, pers. comm.). Following a preculture at 30 °C for 24 h in 5 ml M17 broth, the strains were inoculated at a concentration of 10 ml/l in 20 ml reconstituted skim ewes' milk (130 g/l) that had been sterilized 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 incubation at 30 °C for 24 h, gas production was determined as described by Kihal (1995). The method involves measuring the displacement of a column of water in a 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 carried out 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 2 M-HCl was added to the vessel with a syringe and needle. The volume of water displaced was measured after stirring the culture for 3 min. The $CO_2$ production by cultures was calculated by assuming that one mole of $CO_2$ occupies a volume of 24·04 l at 20 °C and 1 atm (101·325 kPa). We found that a portion of the $CO_2$ remained in the medium in the form of $H_2CO_3$. To determine total $CO_2$, it was necessary to calibrate the system with sodium bicarbonate in milk, when we obtained the following relationship by polynomial regression.

$$B = -0{\cdot}0014A^2 + 1{\cdot}4768A, \qquad (r^2 = 0{\cdot}999)$$

where $A$ is $CO_2$ production (in mM) without correcting for the $CO_2$ remaining in the medium and $B$ is the corrected value.

A linear regression ($B = 1{\cdot}3035A$) would probably be acceptable in practice, but we used the polynomial expression as the linear relationship had a slightly lower $r^2$ (0·995). The CV for the measurements of $CO_2$ production were generally $\sim 3\,\%$.

## RESULTS

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

Many microorganisms reduce TTC to formazan, an insoluble compound that colours colonies red or brown, but TTC is not reduced 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. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 mutants with attenuated LDH activity by varying the buffering capacity of LDHA medium by supplementing it with different quantities of sodium glycerophosphate. At a concentration of 20 g/l, strain CNRZ 483 formed white colonies. Under the same conditions mutant 483L1, whose LDH activity was 9 % that of the parent strain (Boumerdassi *et al*. 1997), formed colonies with a brown centre. Mutant 483L2, whose LDH activity was only 0·3 % that of the parent strain formed colonies that were entirely brown. At a concentration of 40 g glycerophosphate/l, 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

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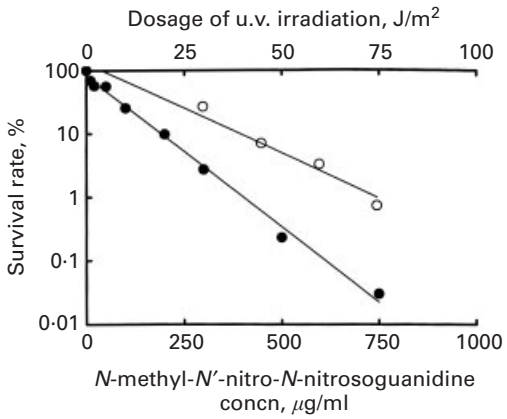



Fig. 1. Effect of ●, the *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine concentration or ○, the dosage of u.v. radiation on the viability of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* RD311 cells.

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

| Mutagen treatment | Survival rate, % | No. of colonies examined | No. of brown colonies | Incidence of brown colonies, % |
|---|---|---|---|---|
| Incubation in the absence of NTG ‡ | 100·0 | $4{\cdot}1 \times 10^5$ | 17 | $4{\cdot}1 \times 10^{-3}$ |
| Incubation with 20 μg NTG/ml | 56·8 | $3{\cdot}5 \times 10^4$ | 124 | $3{\cdot}5 \times 10^{-1}$ |
| Incubation with 200 μg NTG/ml | 9·8 | $1{\cdot}7 \times 10^4$ | 121 | $7{\cdot}1 \times 10^{-1}$ |
| Subject to u.v. irradiation at 44·7 J/m² | 7·0 | $3{\cdot}0 \times 10^5$ | 100 | $3{\cdot}3 \times 10^{-2}$ |

NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.
† For composition and preparation of medium, see text.
‡ Spontaneous mutation.

colonies, whereas mutant 483L2 formed colonies that were entirely brown or had a brown centre. LDHA medium supplemented with 20 g glycerophosphate/l, LDHA-20, was thus used in the remainder of this work to screen for mutants. It enabled us to distinguish a single colony of mutant 483L1 or 483L2 on a Petri dish among $> 1000$ colonies of the parent strain.

*Selection of mutants on LDHA-20 agar*

The effect of the u.v. dose and the NTG concentration on the survival of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* RD311 was determined (Fig. 1). In the case of both mutagens, the logarithm of 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). These were incubation of the cells in the absence of NTG (spontaneous mutation), incubation in the presence of 20 μg NTG/ml, incubation in the presence of 200 μg NTG/ml and after u.v. irradiation at 44·7 J/m². The highest incidence of brown colonies was obtained with 200 μg NTG/ml. This frequency decreased to about half for 20 μg NTG/ml, to $\sim 1/20$ with u.v. treatment and to $\sim 1/200$ for spontaneous mutation.

*Carbon dioxide production*

After each mutagen treatment, mutants forming brown colonies on LDHA-20 agar were ranked according to their $CO_2$ production (Table 2). For the four mutagen treatments, about half the mutants produced between 15 and 30 mM-$CO_2$, the same

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

| | No. of mutants in each range of $CO_2$ production, % | | | |
|---|---|---|---|---|
| $CO_2$ production range, mM | Spontaneous mutants | Mutants forme with 20 μg NTG/ml | Mutants formed with 200 μg NTG/ml | Mutants formed with u.v. irradiation at 44·7 J/m² |
| 0–15 | 0·0 | 4·0 | 14·0 | 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 | 4·0 | 5·0 | 2·0 |
| 75–90 | 0 | 0·8 | 7·4 | 8·0 |
| 90–105 | 5·9 | 7·3 | 2·5 | 5·0 |
| 105–120 | 0 | 10·5 | 0·8 | 5·0 |

NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.
† For composition and preparation of medium, see text.

as or slightly higher than the parent strain, which produced 18 mM. In contrast to spontaneous and u.v.-induced mutants, some of the NTG-induced mutants produced $< 15$ mM-$CO_2$. Some spontaneous mutants produced $> 90$ mM-$CO_2$, whereas some NTG- and u.v.-induced mutants produced $> 105$ mM. Relative to the total number of colonies screened (i.e. the number of white colonies plus the number of brown colonies), the percentages of mutants with $CO_2$ production $> 45$ mM were 0·001, 0·109, 0·165 and 0·008% for spontaneous mutation and mutagenesis with 20 μg NTG/ml, 200 μg NTG/ml and u.v. respectively.

## DISCUSSION

The LDHA-20 medium enabled us to distinguish colonies of the parent strain of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 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 emphasize, however, that TTC is reduced only if the pH is not excessively acid (Lederberg, 1948). Thus on acidification parent strains 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. So far, we have tested 24 different strains of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* on LDHA-20 agar. Of these, 22 exhibited the same behaviour as strain CNRZ 483, forming white colonies on LDHA-20 agar, whereas the 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 strain, 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 made it possible to examine a larger number of colonies per Petri dish (1000 *v.* 150) and did not require treatment of the Petri dishes after the growth of colonies (replica plating on velvet and staining with α-naphthol).

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It was thus possible to select mutants in a cell suspension containing a very low proportion of mutants, for example when screening for spontaneous mutants.

We found that about half the mutants forming brown colonies on LDHA-20 agar produced amounts of $CO_2$ similar to the parent strain. Several types of mutations may produce such strains. For example, mutants having the same acidifying capacity as the parental strain, but an increased reducing capacity, would form brown colonies without producing excess $CO_2$. In addition, 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 $CO_2$. Such mutants would not be of technological interest, which is why it is necessary to add a second stage after selecting mutants on LDHA-20 agar, namely the measurement of $CO_2$ production in milk cultures. The procedure described here has the advantage of being very precise and simple to use in practice.

The present work centred around the development of a method for selecting mutants producing excess $CO_2$. These selected mutants will be studied in relation to their metabolism of lactose and citrate in milk, their LDH activity and their stability.

We thank G. Yonnet for her technical assistance and J.-P. Reverbel (Société des Caves de Roquefort) for his helpful suggestions. This work was partially supported by the European Union (contract AIR3-CT94–2010). A. El Attar is the recipient of a Franco-Egyptian cooperation fellowship from the French Foreign Affairs Ministry.

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