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New insights into *Lactococcus lactis* diacetyl- and acetoin-producing strains isolated from diverse origins

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ABSTRACT

Lactococcus lactis subsp. *lactis* biovar diacetylactis strains are used in the dairy industry for generating acetoin and notably diacetyl which imparts a high level of buttery flavor notes.
A collection of domesticated and environmental strains was screened for the production of diacetyl or acetoin (D/A), and citrate fermentation. Unexpectedly, both domesticated and environmental strains produced D/A. Domesticated strains belonging to the currently named "biovar diacetylactis" metabolized citrate and produced large amounts of D/A during early growth. They harbored the *citP* plasmid gene encoding citrate permease and a chromosomal region *citM-citI-citCDEFXG* involved in citrate metabolism. In these strains, citrate consumption was identified as the major determinant of aroma production. Environmental strains, specifically UCMA5716 and A12, produced as much D/A as the CitP$^+$ strains, though at slightly lower rates. UCMA5716 was found to contain the *citM-citI-citCDEFXG* cluster but not the *citP* gene. A12 had neither. In these strains, production rate of D/A was linearly correlated with pyruvate synthesis rate. However, the correlation factor was strain-dependent, suggesting different modes of regulation for pyruvate rerouting towards fermentation end-products and flavors. This work highlights the genetic and metabolic differences between environmental and domesticated strains. The introduction of environmental strains into industrial processes could considerably increase the diversity of starters, enhancing the delivery of new technological properties.

1. Introduction

Lactococcus lactis subsp. *lactis* biovar diacetylactis is commonly used in the dairy industry for its capacity to produce diacetyl and acetoin. Diacetyl is an essential component of many dairy products since low concentrations provide creamy and buttery aroma. This compound is also a key constituent of Camembert, Cheddar and Emmental (Curioni and Bosset, 2002). In cheese manufacturing, *L. lactis* subsp. *lactis* biovar diacetylactis is generally mixed with other lactic acid bacteria (LAB). It may account for up to 20% of the starter culture, particularly in fresh (soft) cheese, buttermilk, and Gouda- or Edam-type cheeses (Fox and McSweeney, 2004; Smit et al., 2005; Teuber and Geis, 2006; Urbach, 1997).

In *L. lactis* subsp. *lactis* biovar diacetylactis strains, aroma production is associated with the capacity to metabolize citrate, diacetyl production being proportional to citrate consumption in milk (Aymes et al., 1999). The only strain in which citrate metabolism has been investigated in depth is the dairy strain CRL264, isolated from cheese (Sesma et al., 1990). Two clusters of genes have been implicated in citrate metabolism. The plasmid-borne *citQRP* operon is required for citrate uptake. This operon is composed of the *citP* gene, encoding a citrate permease, the *citR* gene, encoding the CitR protein, which regulates the *citR* and *citP* genes, and the *citQ* gene, a putative open reading frame overlapping that of *citR* (López de Felipe et al., 1995). CitP is the only citrate transporter system identified to date in *L. lactis*. It catalyzes the Hcit^{2-} and lactate$^-$ antiport (García-Quintáns et al., 2008; Sobczak and Lolkema, 2005). Other metabolites (pyruvate, α-acetolactate, and acetate) have been recently reported to participate in exchange reactions for citrate uptake (Pudlik and Lolkema, 2011). The formation of pyruvate from citrate involves the chromosomal *citM-citI-citCDEFXG* genes (hereafter referred to as *citM-G*) encoding the α, β, and γ

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citrate lyase (CL) subunits (CitF, CitE, and CitD, respectively), the CitC, CitX, and CitG auxiliary proteins and the CitM oxaloacetate decarboxylase (annoted *mae* in the IL1403 strain). Upstream from *citC*, there is a divergent open reading frame, *citI*, which may be involved in regulating the CitP protein and CL complex assembly (Martin et al., 2005). In a few atypical citrate-fermenting *L. lactis* strains isolated from dromedary's milk, the *citM-G* cluster is not chromosomal, but located on a 23 kb plasmid, upstream from the *citP* gene (Drici et al., 2010).

Descriptions of biovar diacetylactis to date have included only citrate-utilizing strains. Recent investigations of population structure and diversity of *L. lactis* subsp. *lactis* clustered *L. lactis* biovar diacetylactis strains into a single clonal complex with low levels of genetic diversity (Passerini et al., 2010). This clonal complex consists of strains isolated from dairy starters or involved in milk processing, designated as "domesticated" strains. By contrast, "environmental" strains, isolated from animal skin, plants and raw milk, constitute the major reservoir of genetic diversity that has yet to be screened for its diacetyl or acetoin (D/A) production capacity. In this study, we explored the ability of domesticated and environmental *L. lactis* subsp. *lactis* strains to produce D/A, in order to determine whether environmental strains could be considered to belong to the diacetylactis biovar. We analyzed 36 strains by a combination of phenotypic and genotypic approaches. For domesticated or environmental strains with different phenotypes or genotypes, we investigated the determinants of D/A production by carrying out physiological studies. This work provides new insights into the diacetylactis biovar classification.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *L. lactis* subsp. *lactis* strains (Table 1) from national and private French collections used here were characterized in a previous study (Passerini et al., 2010). They were routinely cultured at 30 °C in M17-broth (Merck KGaA, Darmstadt, Germany) supplemented with 5 g/l (w/v) glucose (GM17 broth). Strains were grown in semi-synthetic medium containing 160 mM glucose, 30 mM citrate, 10 g/l yeast extract (Sigma-Aldrich), 9 g/l KH_2PO_4 for flasks (3 g/l for fermentors), 7.5 g/l K_2HPO_4 for flasks (2.5 g/l for fermentors), 0.2 g/l $MgSO_4,7H_2O$, and 0.05 g/l $MnSO_4,H_2O$. Yeast extract has been shown to stimulate bacterial growth and citrate utilization (Monnet et al., 2000). Bacteria were cultured in aerobic conditions, in either 1-liter baffled flasks containing 200 ml of medium, shaken at 100 rpm, or in a 2-liter fermentor (Sartorius, Germany) rotating at 250 rpm. The temperature was maintained at 30 °C and the pH was left unregulated in the flasks (initial value of 6.6). In fermentor cultures, the pH initially rapidly decreased and was then maintained at 5.5 by the automatic addition of KOH (10 N). The fermentors were aerated, such that the pO_2 was maintained at 80%, and the outlet gas phase was flushed with cold water (0 °C) for the quantification of total aroma compounds production.

2.2. Phenotypic characterization

The citrate-fermenting capacity of *L. lactis* strains was analyzed on Kempler and McKay agar (Kempler and McKay, 1980), after 48 h of incubation at 30 °C. In these conditions, the citrate-fermenting strains formed blue colonies (KMK^+ phenotype) whereas strains unable to ferment citrate formed white colonies (KMK^- phenotype).

Acetoin production was detected by the Voges–Proskauer (VP) reaction. Cells were grown in Clark and Lubs medium (5 g/l biopolypeptone; 5 g/l glucose; 5 g/l KH_2PO_4; pH 7.5) at 30 °C. After 24 h, we added 0.5 ml of α-naphthol (6%, w/v ethanol) and 0.5 ml of potassium hydroxide (16%, v/v) to 2 ml of bacterial suspension and incubated the resulting mixture for 1 h. Acetoin production was detected as a red ring on the surface of the culture (VP^+ phenotype) (Speckman and Collins, 1982).

2.3. Biomass and metabolite analysis

Bacterial growth was estimated by spectrophotometric measurements at 580 nm, with a Biochrom Libra S4 spectrophotometer (for *L. lactis*, 1 absorbance unit corresponds to 0.3 g of dry weight/l biomass). The concentrations of glucose, lactate, acetate, citrate, pyruvate, butanediol, acetolactate, acetoin and diacetyl in the culture supernatant and in gas-phase condensate were determined by high-performance liquid chromatography with a 1200 series system (Agilent Technologies, Waldbronn, Germany), as previously described (Cocaign-Bousquet and Lindley, 1995). Briefly, a Bio-Rad $HPX87H^+$ column was maintained at a temperature of 48 °C, and 5 mM H_2SO_4 was used as the eluent, at a flow rate of 0.5 ml min^{-1}. Dual detection was carried out with a refractometer and UV analyses. The amounts of volatile aroma compounds produced in fermentor cultures were calculated by summing the values for the culture supernatants and the gas phase condensate.

2.4. PCR amplification

Genomic DNA was extracted with the "Wizard Genomic DNA purification" kit, in accordance with the manufacturers' protocols (Promega, Madison, USA). The *citP* gene and the *citM-G* region from *L. lactis* were amplified by PCR with the Phusion DNA polymerase (Finnzyme, Vantaa, Finland), in accordance with the manufacturers' instructions, with 0.5 μM of the following primers: citP1 (5′-ATGATGAATCACCCG-3′) and citP2 (5′-ACTTCATGAATATGAC-3′) for *citP* gene amplification; citM_F (5′-ATGAATGCAGCCAAGTTAG-3′) and citG_R (5′-TGATGTGAA CCGTTAGTTAC-3′) for *citM-citG* amplification; citD F (5′-TGATTAAGG CCAGAGCGATT-3′) and citF R (5′-TTTAGCTGTCCCAGCGTCTT-3′) for *citDEF* amplification.

2.5. PFGE, transfer and hybridization

Preparation of lactococcal DNA embedded in agarose matrix, digestion of DNA, pulsed field gel electrophoresis (PFGE), and hybridization with dried agarose gels were performed as previously described (Le Bourgeois et al., 1995). Plasmids were linearized by digestion with S1 nuclease (Barton et al., 1995). Briefly, DNA embedded in an agarose matrix was incubated at 37 °C for 40 min with 2.5 U of S1 nuclease in 200 μl of 1XS1 buffer (Promega, Madison, USA). The reaction was stopped by adding 1 ml of TE 10/50 (10 mM Tris–HCl pH 8; 50 mM EDTA). Linearized plasmids were separated by PFGE, and analyzed by hybridization. Citrate plasmids and the *citM-G* region were identified with the amplified *citP* and *citDEF* fragments labeled with [α-^{32}P] dATP and used as probes.

2.6. Statistical treatment of data

All experiments data were performed at least in duplicate and results are represented as averages ± standard deviations. For flask fermentation, the standard deviation was calculated from the LD61 strain (Raynaud et al., 2005) used as control for each set of culture and performed in triplicate. To determine statistically significant data, Student *t*-tests and one-way analysis of variance (ANOVA) were performed with XLSTAT Excel 2012.1 software using 0.05 *p*-value.

3. Results

3.1. Relationship between gene content and phenotype

We first investigated the presence of the *citM-G* region and the *citP* gene by PCR amplification in 36 strains of *L. lactis* subsp. *lactis*,