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Selection and properties of α-acetolactate decarboxylase-deficient spontaneous mutants of *Streptococcus thermophilus*

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Abstract

Many lactic acid bacteria produce diacetyl, which is a desirable aroma compound in some fermented dairy products. Strains or mutants of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* that are deficient in α-acetolactate decarboxylase are used in some food processes for their ability to produce large amounts of diacetyl. However, up until now, the use of α-acetolactate decarboxylase-deficient mutants of *Streptococcus thermophilus* for increased diacetyl production has not been evaluated. The objective of the present study was to devise a procedure for selecting spontaneous α-acetolactate decarboxylase-deficient mutants of *S. thermophilus*. We observed that in a chemically defined medium (CDM) containing α-ketobutyrate plus leucine, or α-ketobutyrate plus leucine plus isoleucine, the α-acetolactate decarboxylase-deficient mutant TIL865, obtained by directed mutagenesis, grew faster than its parent strain. This property was used for selecting spontaneous α-acetolactate decarboxylase-deficient mutants on agar plates. The resulting mutants were able to grow in milk, and their acidifying activity was slightly lower than that of the parent strain. Under partial anaerobic or aerobic conditions, they produced approximately three times more diacetyl than the parent strain. Such spontaneous mutants may be useful for increasing the diacetyl content of fermented milks whose production involves *S. thermophilus* strains.

Keywords: Streptococcus thermophilus; Lactic acid bacteria; Diacetyl; Milk; Yogurt; Mutation

1. Introduction

Diacetyl is a major flavor compound in many cultured dairy products. It is an end product of citrate metabolism by certain lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* sp., which are used for the production of cultured cream, cultured butter, buttermilk and fresh cheeses. Diacetyl is also a key aroma compound in yogurt (Imhof et al., 1995; Ott et al., 1997), but in this case, it is mainly produced by *Streptococcus thermophilus*, which is a lactic acid bacterium that is not able to use citrate.

Strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis* that are deficient in α-acetolactate decarboxylase, the enzyme responsible for acetoin production, accumulate significant amounts of α-acetolactate in the culture medium. This results in higher diacetyl production due to the spontaneous oxidative decarboxylation of α-acetolactate (De Man, 1959). Such strains are widely used in butter-making processes (Veringa et al., 1976) and for the production of aroma additives with a high α-acetolactate (Kuiper et al., 1987) or diacetyl (Jönsson et al., 1980) content. In *L. lactis* strains isolated from non-dairy environments, α-acetolactate decarboxylase is also involved in the regulation of leucine and valine biosynthesis (Goupil-Feuillerat et al., 1997). Indeed, α-acetolactate is a precursor of these branched-chain amino acids, and when they are present in excess, the flux of α-acetolactate is diverted towards acetoin, due to an allosteric activation of the α-acetolactate decarboxylase (Phalip et al., 1994) and to a translational regulation of α-acetolactate decarboxylase synthesis (Goupil-Feuillerat et al., 2000). Goupil et al. (1996) took advantage of the fact that this enzyme has a central role in the regulation of branched-chain amino acid biosynthesis to select spontaneous α-acetolactate decarboxylase-deficient mutants, by using a defined medium containing leucine, but devoid of valine and isoleucine.

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We recently showed that α-acetolactate decarboxylase is also involved in the regulation of leucine and valine biosynthesis by *S. thermophilus* (Monnet et al., 2003) (Fig. 1). The objective of the present study was to determine whether this property could be used for devising a medium for the selection of spontaneous α-acetolactate decarboxylase-deficient mutants, and whether such mutants were able to produce large amounts of diacetyl in milk.

2. Materials and methods

2.1. Bacterial strains

S. thermophilus strains were routinely grown in M17 broth (Terzaghi and Sandine, 1975). Strain CNRZ385 was obtained from UBLO (Unité Bactéries Lactiques et Pathogènes Opportunistes, INRA, Jouy-en-Josas, France), and strain TIL865 is an α-acetolactate decarboxylase-deficient mutant selected from strain CNRZ385 by directed mutagenesis (Monnet et al., 2003).

2.2. Growth in defined media

α-ketobutyrate on the growth of *S. thermophilus* was studied using a chemically defined medium (CDM), derived from the medium described by Reiter and Oram (1962). It contained lactose (10 g/l), KH_2PO_4 (3 g/l), sodium acetate (1 g/l), ascorbic acid (0.5 g/l), adenine (5 mg/l), guanine (5 mg/l), xanthine (5 mg/l), uracil (5 mg/l), pyridoxal hydrochloride (2 mg/l), niacin (1 mg/l), thiamine hydrochloride (1 mg/l), riboflavin (1 mg/l), calcium pantothenate (1 mg/l), para-aminobenzoic acid (10 μg/l), biotin (10 μg/l), folic acid (1 μg/l), vitamin B12 (1 μg/l), $MgCl_2 \cdot 6H_2O$ (200 mg/l), $CaCl_2$ (50 mg/l), $FeCl_3 \cdot 6H_2O$ (5 mg/l), $ZnSO_4 \cdot 7H_2O$ (5 mg/l), $CoCl_2 \cdot 6H_2O$ (2.5 mg/l), $CuSO_4 \cdot 5H_2O$ (2.5 mg/l), $NiSO_4 \cdot 7H_2O$ (10 mg/l), $MnSO_4 \cdot 1H_2O$ (10 mg/l), L-cysteine hydrochloride (200 mg/l), L-alanine (150 mg/l), L-lysine hydrochloride (800 mg/l), L-arginine (450 mg/l), L-proline (540 mg/l), L-methionine (70 mg/l), L-phenylalanine (100 mg/l), L-serine (200 mg/l), L-threonine (100 mg/l), L-tryptophane (100 mg/l), glycine (200 mg/l), L-hystidine hydrochloride $\cdot 1H_2O$ (550 mg/l), L-glutamate (3.2 g/l), L-glutamine (3.2 g/l), L-aspartate (300 mg/l) and L-tyrosine (100 mg/l). The medium was reconstituted using concentrated stock solutions containing the nutrients. A mixture containing lactose, salts and vitamins was prepared at a concentration double that in the CDM. After adjusting its pH to 6.8 using NaOH, the mixture was autoclaved for 15 min at 110 °C. The amino acids were prepared as a five times concentrated solution that was filter-sterilized (0.22 μm) after adjustment of its pH to 6.8. Supplementation of CDM with L-leucine, L-isoleucine, L-valine or sodium α-ketobutyrate was done at a final concentration of 200, 100, 150 and 600 mg/l, respectively. These compounds were prepared individually

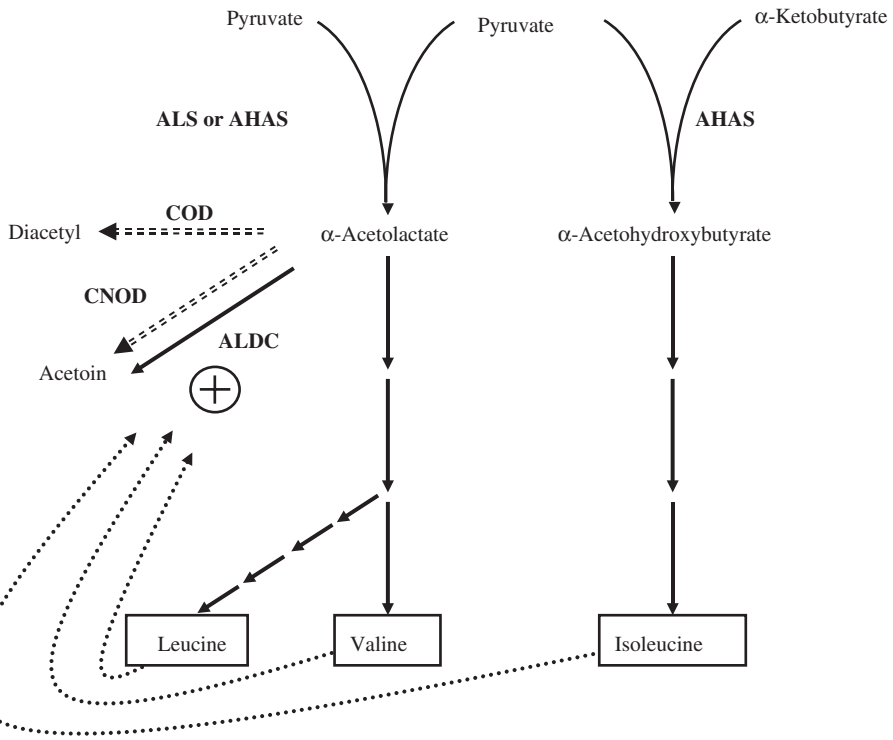

Fig. 1. Schematic representation of the pathways involved in diacetyl and branched-chain amino acid biosynthesis by *Streptococcus thermophilus*. Continuous arrows indicate enzymatic steps, dotted double arrows indicate non-enzymatic steps, and dotted simple arrows indicate allosteric activations. ALS, α-acetolactate synthase; AHAS, acetohydroxy acid synthase; ALDC, α-acetolactate decarboxylase; COD, chemical oxidative decarboxylation; CNOD, chemical non-oxidative decarboxylation.