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ORIGINAL PAPER

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Effect of *ilvBN*-encoded α-acetolactate synthase expression on diacetyl production in *Lactococcus lactis*

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Abstract Conversion of pyruvate to α-acetolactate, which is broken down to diacetyl and acetoin, can be catalysed by two α-acetolactate synthases in *Lactococcus lactis*. The enzyme encoded by the *als* gene (Als) has previously been shown to have a low affinity for pyruvate, which limits the formation of diacetyl. In this study we have expressed from a plasmid the *ilvBN* genes, which encode the other α-acetolactate synthase (IlvBN). This plasmid-directed enzyme expression provided up to 3.6-fold increased product formation in the *L. lactis* MG1363 and IL1403 backgrounds. Plasmid-based expression of the *ilvBN* genes, in an IL1403 derivative from which the *leu.ilv.ald* and flanking genes had been deleted, yielded up to 0.1 mM diacetyl whereas the host strain produced none. In addition, IlvBN, with a K_m value of 8.3 mM, was shown to have a greater affinity for pyruvate than does Als.

Introduction

Diacetyl is a desirable flavour component of dairy products such as butter and cheeses. The compound is produced via aerobic pyruvate metabolism by certain strains of *Lactococcus lactis* and other lactic acid bacteria. In *L. lactis*, α-acetolactate synthase (EC 4.1.3.18) can convert pyruvate to α-acetolactate. This unstable intermediate is then broken down to diacetyl via oxidative decarboxylation or to acetoin by α-acetolactate decarboxylase (EC 4.1.1.5; Hugenholtz 1993). In *L. lactis* the enzyme diacetyl reductase catalyses the reduction of vicinal diketones and the subsequent reversible reduction of the monohydroxy carbonyl products (Gibson et al. 1991). This enzyme can convert diacetyl to acetoin and acetoin to 2,3-butanediol. Industrial production of dairy products could be improved by the construction of *L. lactis* strains with increased yields of diacetyl.

The α-acetolactate synthase (Als) normally active in the diacetyl production pathway has a low affinity for pyruvate (Snoep et al. 1992; Monnet et al. 1994): pyruvate is not diverted into this pathway unless it is present in excess, e.g. during citrate fermentation by *L. lactis* subsp. *lactis* biovar diacetylactis (Hugenholtz 1993). The *als* gene encoding this enzyme has recently been characterized by Marugg et al. (1994). The *ilvBN* genes proposed to encode another α-acetolactate synthase (IlvBN) were identified in *L. lactis* subsp. *lactis* (Godon et al. 1992). This isozyme's properties have not been characterized. Enzymes homologous to IlvBN (in both prokaryotic and eukaryotic cells) are active in branched-chain amino acid biosynthesis (Umbarger 1987). Also known as acetohydroxy-acid synthases, they can convert two pyruvates to α-acetolactate during either leucine or valine biosynthesis or convert pyruvate plus 2-oxobutyrate to 2-acetohydroxybutyrate during isoleucine biosynthesis. IlvBN may be regulated at two levels: (a) the activity of some homologous enzymes in *E. coli* is regulated by valine via feedback inhibition (Weinstock et al. 1992); (b) expression of the *L. lactis* genes within the *ilv* operon appears to be transcriptionally regulated (Godon et al. 1993). These genes are not transcribed when amino acids are available, e.g. during growth of *L. lactis* in milk (Godon et al. 1993). The *ilv* genes may be inactive in many *L. lactis* strains which are auxotrophic for branched-chain amino acids (Chopin 1993).

In order to increase the level of diacetyl produced by *L. lactis* strains we have generated a plasmid construct

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in which the *ilvBN* genes are expressed from a heterologous promoter. Such *ilvBN* expression (no longer under the operon's transcriptional regulation) produces an α-acetolactate synthase that is found to be active in the presence of branched-chain amino acids in the medium. The plasmid-directed expression of *ilvBN* leads to increased generation of diacetyl and acetoin in several *L. lactis* strains.

Materials and methods

Organisms and growth conditions

Escherichia coli strain MC1022 (Casadaban and Cohen 1980) was used as the recipient in the cloning experiments. The *L. lactis* strains used were MG1363, a plasmid-free *L. lactis* subsp. *cremoris* strain (Gasson 1983), IL1403, a plasmid-free *L. lactis* subsp. *lactis* strain (Chopin et al. 1984), and JIM4882, a derivative of IL1403 from which the chromosomal *leuABCDorf19ilvDBNCAaldBR* genes were deleted (Delorme et al. 1994; J.-J. Godon and P. Renault, unpublished results). *E. coli* strains were propagated in L broth (Miller 1972); *L. lactis* strains were propagated in M17 broth (Terzaghi and Sandine 1975) supplemented with 0.5% (w/v) glucose. When appropriate, media were supplemented with erythromycin (1 mg ml^{-1} for *E. coli*, 10 μg ml^{-1} for *L. lactis*). Batch cultures of *L. lactis* were grown for 16 h at 30°C. Static cultures were grown in 10-ml volumes in 25-ml bottles without shaking; aerated cultures of 10-ml volumes in 100-ml bottles were shaken at 300 rpm.

Plasmids and genetic manipulations

Restriction endonuclease and DNA-modification enzymes were obtained from Gibco BRL, Paisley, UK, and were used according to the manufacturers instructions. Plasmid pMG36 is a lactococcal vector encoding kanamycin-resistance (Van de Guchte 1991). Plasmid pMG36e is a derivative of pMG36 in which the kanamycin-resistance determinant has been replaced with an erythromycin-resistance gene (Van de Guchte 1991). The nucleotide sequence of the promoter from this plasmid has been published previously (van der Vossen et al. 1987). Plasmid pMTL23 is an *E. coli* cloning vector (Chambers et al. 1988). Plasmids pIL523 and pIL525 are derivatives of plasmid pIL500: pIL500 contains DNA cloned from *L. lactis* subsp. *lactis* NCDO2118 (Godon et al. 1992; J.-J. Godon and P. Renault, unpublished results). pIL523 contains the start of the *ilvB* gene up to an *Eco*RI site, while pIL525 incorporates the rest of the *ilv* operon downstream of the *Eco*RI site (Fig. 1). A 4.5-kb *Eco*RI-*Bam*HI fragment encoding *ilvBNCA* was excised from pIL525 and inserted into the vector pMTL23 to produce plasmid pFI686 (Fig. 1). A 205-bp section of the start of *ilvB* was amplified by the polymerase chain reaction (PCR) from pIL523 with a *Bgl*II site added upstream of the ribosome-binding site. After digestion with *Bgl*II and *Eco*RI the fragment was inserted between the *Bgl*II site of the polylinker of pFI686 and the *Eco*RI site at the upstream end of the *ilvBNCA* fragment, to produce pFI687. Sequence analysis of pFI687 confirmed that the sequence of the PCR segment is correct. Two adjacent *Hin*cII fragments encoding most of *ilvC* were replaced by a 1.2-kb *Hin*cII fragment of the gene for kanamycin resistance from pUC-4 K (Pharmacia), yielding pFI688. Finally, pFI688 was cut with *Bgl*II, the ends blunted, and *Sst*I linkers added. Further digestion with *Sal*I (which cuts at the 3′ end of the gene for kanamycin resistance) generated a 3.3-kb *ilvBN*-Kan^R fragment, which was inserted between the *Sst*I and *Sal*I sites of the vector pMG36e downstream of the promoter P32, yielding pFI749 (Fig. 1). Standard molecular cloning, PCR, transformation, and electrophoresis techniques were used (Griffin and Griffin 1995; Sambrook et al. 1989). Electroporation of *L. lactis* was performed as described by Holo and Nes (1989).

Determination of pyruvate metabolites

Cultures were centrifuged at 17 350 g for 2 min and the supernatants analysed. α-Acetolactate and acetoin levels were measured colorimetrically on the basis of the method of Westerfeld (1945). A 200-μl sample of supernatant and 400 μl 1 M NaOH or 0.5 M HCl

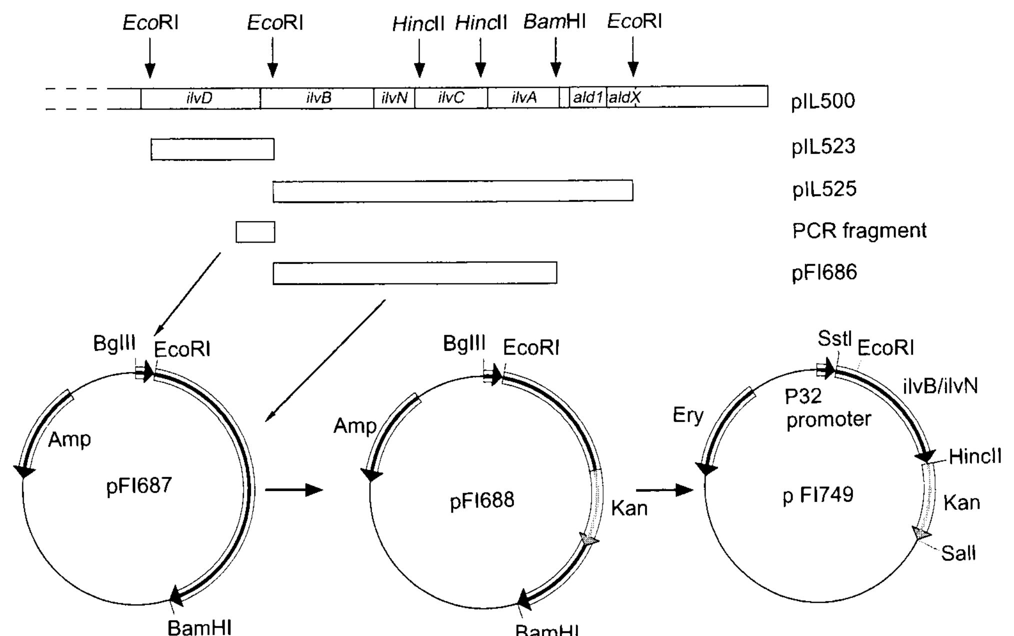

Fig. 1 Construction of plasmid pFI749 incorporating the α-acetolactate-synthase-encoding genes (*ilvBN*) downstream of the lactococcal promoter P32

were heated at 44°C for 30 min; the solutions were then made up to 5 ml with H_2O, creatine and α-naphthol were added, and the tubes were incubated at 20°C for 60 min before the absorption was read at 525 nm. The concentration of α-acetolactate was obtained by subtraction of the acetoin concentration (determined in the NaOH-treated samples) from the total concentration of decarboxylated α-acetolactate plus acetoin (determined in the HCl-treated samples) and the result multiplied by 100/62 (Jordan and Cogan 1988; 62% of α-acetolactate standard was converted to acetoin under the assay conditions). Diacetyl production was measured colorimetrically on the basis of the method of Pien et al. (1937). A 680-μl aliquot of supernatant and 20 μl 0.5% 3,3-diaminobenzidine tetrahydrochloride were incubated in the dark for 1 min at room temperature; 200 μl 3 M H_2SO_4 and 100 μl H_2O were added and the tubes were incubated in the dark for 10 min at room temperature before the absorption was read at 366 nm. The absorption of broth treated similarly was subtracted from the absorption of each sample.

Results

Affinity for pyruvate of the *ilvBN*-encoded α-acetolactate synthase

The wild-type *L. lactis* strain NCDO2118 was used as a source of the *ilvBN* genes (Godon et al. 1992) since, unlike most dairy strains, it is prototrophic. Plasmid pFI749 was constructed such that the *ilvBN* genes from NCDO2118 are transcribed from the lactococcal promoter P32 (van der Vossen et al. 1987) and followed by a transcription terminator (Fig. 1; see Materials and methods). Expression of *ilvBN* from pFI749 produced α-acetolactate synthase activity in a *L. lactis* strain lacking this activity as a result of a genomic deletion (data not shown).

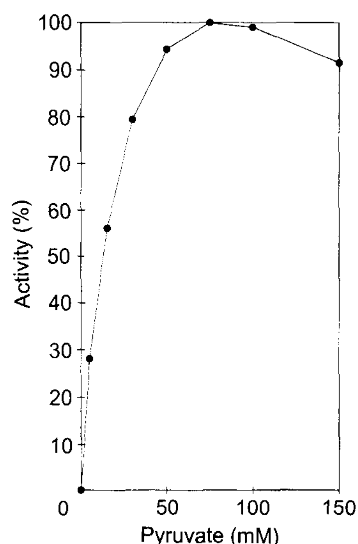

Fig. 2 Saturation kinetics for pyruvate of *ilvBN*-encoded α-acetolactate synthase

Expression of *ilvBN* in the absence of background α-acetolactate activity allowed characterization of the affinity of IlvBN for pyruvate. The enzyme displayed Michaelis-Menten kinetics for substrate concentrations up to 100 mM (Fig. 2), and 75 mM pyruvate was found to be the optimum substrate concentration for IlvBN activity. Construction of a Lineweaver-Burk plot provided a K_m value of 8.3 mM (data not shown).

Product formation with and without the *ilvBN* expression vector

pFI749 was used to express *ilvBN* in *L. lactis* strains MG1363, IL1403, and JIM4882 (a derivative of IL1403 from which the chromosomal *ilv* and the flanking *leu* and *ald* operons have been deleted; J.-J. Godon and P. Renault, unpublished results). Strains harbouring plasmid pFI749 demonstrated increased production of α-acetolactate, acetoin and diacetyl (Table 1). The product levels increased up to 3.6-fold in the MG1363 and IL1403 backgrounds. IL1403 produced higher levels of acetoin and diacetyl than did MG1363. However, no acetoin or diacetyl production could be detected in JIM4882 (Table 1). JIM4882 cells containing plasmid pFI749 yielded 0.1 mM diacetyl. α-Acetolactate was detected in the JIM4882(pFI749) cultures presumably through the deletion of *aldB*, which encodes α-acetolactate decarboxylase (EC 4.1.1.5). The low levels of acetoin produced by JIM4882(pFI749) probably result from chemical decarboxylation of α-acetolactate or conversion of diacetyl by diacetyl reductase. Higher levels of α-acetolactate and diacetyl were produced by the pFI749-containing strains during aerated growth (Table 1).

Table 1 Concentrations of pyruvate metabolites after 16 h growth by strains with or without pFI749. *ND* not detected

	α-Acetolactate (mM)		Acetoin (mM)		Diacetyl (mM)	
Strain	Static	Aerated	Static	Aerated	Static	Aerated
MG1363	ND	ND	ND	0.85	ND	ND
MG1363(pFI749)	ND	ND	ND	3.08	ND	0.02
IL1403	ND	ND	ND	1.28	ND	0.05
IL1403(pFI749)	ND	ND	ND	3.25	0.03	0.08
JIM4882	ND	ND	ND	ND	ND	ND
JIM4882(pFI749)	0.30	0.55	0.10	0.21	0.03	0.10

Discussion

Plasmid-directed expression of the *ilvBN*-encoded α-acetolactate synthase generated increased production of diacetyl and acetoin. Product formation increased up to 3.6-fold in the MG1363 and IL1403 backgrounds. The results presented here confirm that the genes identified as *ilvBN* by Godon et al. (1992) encode an α-acetolactate synthase. The plasmid-encoded *ilvBN* genes are expressed and the enzyme is active in the presence of branched-chain amino acids in the medium. Clearly, the IlvBN isozyme can be used to divert pyruvate into the diacetyl production pathway.

Marked differences in acetoin and diacetyl production between static and aerated cultures were detected (Table 1). Previous studies have noted that the production of these compounds increases 3- to 9-fold when the culture medium is agitated (Bassit et al. 1993). This may be due to the increased activities of α-acetolactate synthase and NADH oxidase in aerated conditions (Bassit et al. 1993), or to decreased pyruvate-formate lyase activity in these conditions (Starrenburg and Hugenholtz 1991).

The saturation kinetics of IlvBN demonstrate that this enzyme has a greater affinity for pyruvate than does the purified Als of *L. lactis* subsp. *lactis* biovar diacetylactis strain C17: IlvBN is shown here to have a K_m value of 8.3 mM compared to the K_m value of 50 mM for Als (Snoep et al. 1992). A greater affinity for the substrate should allow greater generation of diacetyl from pyruvate. Greater substrate affinity may also allow diversion of pyruvate to the diacetyl production pathway during static growth in the presence of pFI749. In wild-type cultures, static growth allows increased activity of other enzymes (e.g. lactate dehydrogenase, pyruvate/formate lyase), reducing the pool of pyruvate available to α-acetolactate synthase isozymes. Als may not be able to act on this lower concentration of pyruvate but IlvBN is able to convert some of the pyruvate.

Summation of the concentrations of α-acetolactate, diacetyl and acetoin that were formed by JIM4882(pFI749) reveals that the total (0.86 mM) was less than the increase in product formation (2.0 mM) upon expression of pFI749 in IL1403. It is possible that, in JIM4882(pFI749) cultures, diacetyl/acetoin reductase is more active in the conversion of diacetyl and acetoin to 2,3-butanediol, since this enzyme is inhibited by an acetoin concentration greater than 1 mM as in IL1403(pFI749) cultures (Hugenholtz 1993).

MG1363 and IL1403 must express an active α-acetolactate decarboxylase since the majority of the α-acetolactate produced by the α-acetolactate synthase is converted to acetoin. The elimination of α-acetolactate decarboxylase activity in JIM4882 (pFI749) provides evidence that the decarboxylase present in IL1403 is encoded by the *aldB* gene.

Diacetyl, an important flavour additive in the food industry, is currently produced by fermentation of a strain of *L. lactis* subsp. *lactis* biovar. diacetylactis. The genetic manipulation of metabolic pathways in *L. lactis*, described in this paper, could lead to improved yields of diacetyl and has considerable significance to the dairy and biotechnology industries.

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