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NOTES

Genetic Manipulation of the Pathway for Diacetyl Metabolism in *Lactococcus lactis*

SIMON R. SWINDELL,[1] KIM H. BENSON,[1] HUGH G. GRIFFIN,[1]* P. RENAULT,[2] S. DUSKO EHRLICH,[2] AND MICHAEL J. GASSON[1]

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom,[1] *and Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, 78352 Jouy en Josas Cedex, France*[2]

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Diacetyl is an important food flavor compound produced by certain strains of citrate-metabolizing lactic acid bacteria. Citrate is converted to pyruvate, from which diacetyl is produced via intermediate α-acetolactate. This paper reports the cloning and analysis of the gene (*aldB*) encoding α-acetolactate decarboxylase from *Lactococcus lactis* MG1363. Deletion of the MG1363 chromosomal *aldB* gene was achieved by double crossover homologous recombination. The mutant strain was found to produce diacetyl; α-acetolactate decarboxylase activity was eliminated. Overexpression of the cloned *ilvBN* genes (encoding an α-acetolactate synthase) in the *aldB* deletion strain produced even higher levels of α-acetolactate, acetoin, and diacetyl.

Lactic acid bacteria, such as *Lactococcus lactis*, are important in the manufacture of fermented food products from raw agricultural materials such as milk, meat, vegetables, and cereals. These bacteria convert sugars to lactic acid via pyruvate; certain strains that are used in the dairy industry, including *L. lactis* subsp. *lactis* biovar diacetylactis, ferment citrate that is also converted to pyruvate. These strains metabolize pyruvate by an additional pathway that leads to the formation of the flavor compound diacetyl (Fig. 1). The unstable intermediate α-acetolactate can be converted to acetoin by α-acetolactate decarboxylase or by chemical decarboxylation (10). α-Acetolactate can undergo oxidative decarboxylation to form diacetyl. Increased levels of acetoin and diacetyl are found under aerated conditions (1). This may be due in part to the increased activities of α-acetolactate synthase and NADH oxidase (1) or to decreased pyruvate-formate lyase activity (17).

L. lactis MG1363 (4) does not ferment citrate and does not normally produce diacetyl. Here we report the manipulation of genes (*aldB* and *ilvBN*) that encode enzymes associated with the diacetyl metabolic pathway (α-acetolactate decarboxylase and α-acetolactate synthase, respectively), in order to achieve diacetyl production in this strain. In a companion paper (7) we describe the inactivation of the *aldB* gene of prototrophic strains of *L. lactis* subsp. *lactis* biovar diacetylactis, making use of the leucine inhibition phenotype.

L. lactis strains were propagated in M17 broth supplemented with 0.5% (wt/vol) glucose at 30°C as described previously (2). When appropriate, media were supplemented with erythromycin (10 μg/ml for *L. lactis*). 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.

Determination of enzyme activities. After 5.5 h of static growth, cells were harvested by centrifugation at 17,350 × *g* for 2 min and concentrated 20-fold in 50 mM potassium phosphate (pH 6). The cells were disrupted by ultrasonication at 0°C six times for 30 s each, with 2-min interruptions. Unbroken cells and debris were removed by centrifugation at 17,350 × *g* for 2 min. The protein concentration was determined by the method of Bradford (3), with bovine serum albumin as a standard. α-Acetolactate was formed from α-methyl-α-acetoxyethyl acetoacetate (Oxford Chemical Ltd., Brackley, Northants, United Kingdom) by adding two equivalents of NaOH (11). The reaction was carried out at room temperature for 25 min immediately before use. α-Acetolactate decarboxylase activity was assayed in 50 mM potassium phosphate (pH 6)–10 mM α-acetolactate at 30°C; half of the tubes also contained 10 mM leucine. The reaction was started by the addition of 2 to 6 mg of cell extract. After 5 min, the reaction was stopped by the addition of NaOH. Acetoin levels were determined colorimetrically by the method of Westerfeld (21). The solutions were made up to 5 ml with H_2O, creatine and naphthol were added, and the tubes were incubated at 20°C for 60 min before the absorption was read at 525 nm.

Determination of pyruvate metabolites. After 16 h of growth, the cultures were centrifuged at 17,350 × *g* for 2 min, and the supernatants were analyzed. α-Acetolactate and acetoin levels were determined. 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 was multiplied by 100/62; 62% of the α-acetolactate standard was converted to acetoin under the assay conditions as described previously (11). Diacetyl production was determined colorimetrically by the method of Pien et al. (14). The supernatant (680 μl) and 20 μl of 0.5% 3,3-diaminobenzidine tetrahydrochloride were incubated in the dark for 1 min at room temperature; 200 μl of 6 N H_2SO_4 and 100 μl of H_2O were added,

* Corresponding author. Mailing address: Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom. Phone: 44-1603-255180 or 44-1603-255000. Fax: 44-1603-255180 or 44-1603-507723. Electronic mail address: HUGH.GRIFFIN@BBSRC.AC.UK.

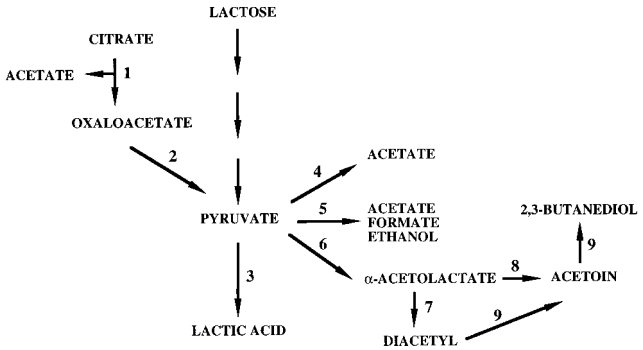

FIG. 1. Pathway for diacetyl metabolism in *L. lactis*. Enzyme or step: 1, citrate lyase; 2, oxaloacetate decarboxylase; 3, lactate dehydrogenase; 4, pyruvate dehydrogenase; 5, pyruvate formate lyase; 6, α-acetolactate synthase; 7, oxidative decarboxylation; 8, α-acetolactate decarboxylase; 9, diacetyl-acetoin reductase.

and the tubes were incubated in the dark for 10 min at room temperature before the absorption was read at 366 nm.

Cloning and sequence analysis of the *aldB* gene from *L. lactis* MG1363. Oligonucleotide primers for use in a PCR were designed from the sequence in the region of the *aldB* gene of *L. lactis* IL1403 (15): primer 1, GGATCCTTACGAACTTTT GTCAGTG, and primer 2, GCGCCAATTTCAACAGTACC ATTATC. These primers were subsequently used to identify individual clones containing the *aldB* gene from a lambda library of MG1363 by the direct PCR-screening method (8, 9). Two positive clones were identified in this way. The insert fragments from these lambda clones were excised by coinfection of *Escherichia coli* with an f1 helper phage that provides enzymes to nick, replicate, and recircularize the pBluescript DNA (Stratagene, Cambridge, United Kingdom). The circularized, single-stranded DNA was reintroduced into *E. coli*, where it was maintained as plasmid DNA. One plasmid, pFI937, was found to have an insert of about 4.2 kbp and was chosen for further study. The DNA sequence of the *aldB* gene of MG1363 was determined.

Deletion of the chromosomal *aldB* gene. PCR primers were designed to amplify the regions flanking the *aldB* gene. The two 1.4-kbp flanking fragments produced were cloned into the *Bam*HI site of pG+host6 (Appligene, Chester-le-Street, United Kingdom) to form pFI805. The insert of plasmid pFI805 is, in effect, a region of lactococcal chromosomal DNA lacking the entire *aldB* gene and can be used to effect a double crossover recombination event that results in a *L. lactis* strain containing an *aldB* deletion.

L. lactis MG1363 was transformed with plasmid pFI805 at the permissive temperature (28°C) with selection for erythromycin. The temperature was then shifted to the restrictive temperature (37°C) at which plasmid replication is disabled. Subsequent growth on erythromycin enabled the selection of cells in which the plasmid had integrated into the chromosome (single crossover integration). Further growth of these strains at 28°C enabled the detection of erythromycin-sensitive cells in which the second crossover (excision) event had occurred. PCR analysis was employed to identify double crossover recombinants containing an *aldB* deletion and to confirm the chromosomal structure. One of the recombinants, strain FI8076, was chosen for further study.

MG1363 was found to express a level of α-acetolactate decarboxylase activity similar to the levels expressed by other *L. lactis* strains (Table 1) (12, 13). α-Acetolactate decarboxylase activity was stimulated 10-fold by the addition of leucine, as observed previously (12, 13). No α-acetolactate decarboxylase activity was detected, in the absence or presence of leucine, in FI8076 cultures. FI8076 produced both α-acetolactate and diacetyl, in contrast to MG1363, which produced only acetoin (Table 1).

Expression of *ilvBN* genes in an *aldB* deletion strain. The *ilvBN* genes express an α-acetolactate synthase and form part of the operon involved in the biosynthesis of isoleucine, leucine, and valine. These genes have been cloned from *L. lactis* (5) and subcloned on a plasmid, pFI749 (2). pFI749 is based on the lactococcal vector pMG36e (19) and contains the cloned *ilvBN* genes expressed from the lactococcal P32 promoter (20) and the erythromycin resistance genetic marker. *L. lactis* FI8076 was transformed with pFI749 DNA, and assays for end metabolites were performed. After aerated growth, the levels of diacetyl, α-acetolactate, and acetoin detected in the FI8076(pFI749) cultures were higher than those found in the plasmid-free FI8076 cultures (Table 1). FI8076(pFI749) also produced α-acetolactate during static growth.

Diacetyl is an important flavor compound in the dairy industry, providing the characteristic buttery flavor of many fermented milk products. Diacetyl is normally produced by a limited number of strains of lactic acid bacteria, such as *L. lactis* subsp. *lactis* biovar diacetylactis, which normally depends on citrate utilization for the production of diacetyl (10). The results presented here demonstrate that citrate utilization and the use of *L. lactis* subsp. *lactis* biovar diacetylactis strains are not essential for diacetyl production. *L. lactis* subsp. *cremoris* MG1363 does not normally utilize citrate or produce diacetyl but does, on aeration, produce acetoin (Table 1). Deletion of the *aldB* gene expressing α-acetolactate decarboxylase increases the cellular pool of α-acetolactate. This α-acetolactate is then available for oxidative decarboxylation to diacetyl. Deletion of the *aldB* gene enables lactococcal strains to produce diacetyl in the absence of citrate utilization (Table 1).

The α-acetolactate synthase that converts pyruvate to α-acetolactate during fermentation of citrate is a single-unit enzyme with a low affinity for pyruvate (12, 16). Recently, the genes (*ilvBN*) encoding another lactococcal acetohydroxy acid synthase (IlvBN) have been described (5). Enzymes homologous to IlvBN convert pyruvate to α-acetolactate during branched-

TABLE 1. α-Acetolactate decarboxylase activity and concentrations of pyruvate metabolites by *aldB*$^+$ or *aldB* strains with or without the *ilvBN* plasmid pFI749

Strain	α-Acetolactate (mM)		Acetoin (mM)		Diacetyl (mM)		α-Acetolactate decarboxylase activity (nmol/min/mg)	
	Static	Aerated	Static	Aerated	Static	Aerated	Without leucine	With leucine
MG1363	0	0	0	0.85	0	0	2	20
MG1363(pFI749)	0	0	0	3.08	0	0.02	2	28
FI8076	0	0.57	0	0.30	0	0.32	0	0
FI8076(pFI749)	0.15	1.48	0	0.73	0	0.53	0	0

chain amino acid biosynthesis (18). Transcript analysis has shown that the *ilvBN* operon is not transcribed when branched-chain amino acids are supplied in the medium, e.g., during growth in milk (6). IlvBN consists of two subunits and has a high affinity for pyruvate (2, 5). The lactococcal P32 promoter has been employed to direct the transcription of the cloned *ilvBN* genes on a plasmid (pFI749). FI8076 (*aldB* deletion strain) harboring pFI749 was shown to produce elevated levels of acetoin and diacetyl as well as α-acetolactate (Table 1). Increased levels of α-acetolactate synthase activity were also found in this strain (data not shown). Increasing the amount of α-acetolactate synthase in the *aldB* deletion strain increases the amount of cellular α-acetolactate. Without an enzymatic conversion to acetoin, much of this α-acetolactate is converted to diacetyl. The increased levels of acetoin in this strain are presumably due to diacetyl reductase-mediated conversion of diacetyl to acetoin or chemical conversion of α-acetolactate to acetoin (Table 1; Fig. 1).

The level of diacetyl (0.53 mM) achieved by a combination of *aldB* deletion and *ilvBN* increased expression compares favorably with the level produced by *L. lactis* subsp. *lactis* biovar diacetylactis (0.087 to 0.17 mM) from citrate fermentation (1, 7). Greater diacetyl production has been reported only for citrate fermentation by a natural mutant lacking α-acetolactate decarboxylase activity (7, 11). In a companion paper (7), *aldB* mutations in *L. lactis* subsp. *lactis* biovar diacetylactis strains increased diacetyl production 10-fold to 0.84 mM. A combination of *aldB* deletion and *ilvBN* increased expression in an *L. lactis* subsp. *lactis* biovar diacetylactis strain utilizing citrate should produce an even greater level of diacetyl. In addition, diacetyl can now be produced by non-citrate-utilizing strains, thus greatly increasing the range of diacetyl-producing strains available for dairy fermentations. Engineering biochemical pathways in this manner is an important method of increasing desirable metabolic products of microbial fermentations used in the food industry. This strategy can also be used to decrease or eliminate the production of nondesirable products of metabolism.

Nucleotide sequence accession number. The nucleotide sequence of the *aldB* gene of MG1363 reported in this paper has been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data banks and has been assigned accession no. X82620.

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