Isolation of the *patC* gene encoding the cystathionine β -lyase of *Lactobacillus delbrueckii* subsp. *bulgaricus* and molecular analysis of inter-strain variability in enzyme biosynthesis

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The *patC* gene encoding the cystathionine β -lyase (CBL) of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCDO 1489 was cloned and expressed in *Escherichia coli*. Overexpression of CBL complemented the methionine auxotrophy of an *E. coli metC* mutant, demonstrating *in vivo* that this enzyme functions as a CBL. However, PatC is distinguishable from the MetC CBLs by a low identity in amino acid sequence, a sensitivity to iodoacetic acid, greater thermostability and a lower substrate affinity. Homologues of *patC* were detected in the 13 *Lb. delbrueckii* strains studied, but only seven of them showed CBL activity. In contrast to CBL⁺ strains, all CBL-deficient strains analysed were auxotrophic for methionine. This supports the hypothesis that CBLs from lactobacilli are probably involved in methionine biosynthesis. Moreover, the results of this study suggest that post-transcriptional mechanisms account for the differences in CBL activities observed between strains of *Lb. delbrueckii*.

Keywords: amino acid catabolism, β -cystathionase, PatC, lactic acid bacteria, cheesemaking

INTRODUCTION

Lactic acid bacteria contribute to flavour formation and texture development in dairy products and are therefore widely used in the dairy industry. Flavour development in cheese is a complex process which is initiated by proteolytic degradation of milk caseins mediated by rennet and the proteases produced by lactic acid bacteria. Subsequently, the aromatic, branched-chain and sulfur-containing amino acids are converted to volatile compounds by chemicals or enzymes which display deaminase, decarboxylase or transaminase activities, or which modify amino acid side chains. Among numerous chemicals, the volatile sulfur compounds methanethiol, dimethyldisulfide (DMDS), dimethyltrisulfide (DMTS) and methional have been found to be

responsible for cheese flavour and aroma (Weimer *et al.*, 1999). Indeed, these methionine breakdown products have been discovered in various cheese types (Urbach, 1993; Barbieri *et al.*, 1994; Engels *et al.*, 1997). The mechanisms by which these compounds arise have been particularly studied in the genus *Lactococcus*, with the ultimate goal of improving cheese flavour.

Cystathionine β -lyase (CBL) (EC 4.4.1.8, also commonly referred to as β -cystathionase) is a pyridoxal-5'-phosphate (PLP)-dependent enzyme which catalyses mainly the cleavage of L-cystathionine to homocysteine, pyruvate and ammonia via an α/β -elimination reaction; that is, the enzyme splits the α C–N (deaminating) and β C–S bonds (Clausen *et al.*, 1997). Subsequently, the homocysteine is methylated by the homocysteine methyltransferase to form methionine (Weimer *et al.*, 1999). However MetC, the purified CBL, from *Lactococcus lactis* subsp. *cremoris* B78 or MG1363 is also able to degrade cystathionine to cysteine, α -ketobutyrate and ammonia, or to degrade methionine (γ C–S linkage) in methanethiol via an α/γ -elimination reaction (Alting *et*

Abbreviations: CBL, cystathionine β -lyase; DMDS, dimethyldisulfide; DMTS, dimethyltrisulfide; PLP, pyridoxal 5'-phosphate.

The GenBank accession number for the sequence determined in this work is AF423071.

al., 1995; Dobric *et al.*, 2000). In a following reaction, the methanethiol can be oxidized to DMDS or DMTS (Parliament *et al.*, 1982). However, no significant differences in the amount of DMDS formed were found between the MG1363 parental strain and the *metC* mutant, suggesting that other enzymes may play a more substantial role in the conversion of methionine (Fernandez *et al.*, 2000). In fact, the cystathionine γ -lyase (Bruinenberg *et al.*, 1997), the aromatic aminotransferase AraT (Yvon *et al.*, 1997; Rijnen *et al.*, 1999), and the branched-chain aminotransferases AT-A and AT-B (Engels *et al.*, 2000) or ILvE (Atilas *et al.*, 2000) have also been reported to contribute to the conversion of methionine to flavour compounds.

Although the sulfur-containing amino acid catabolism of lactococci is well described, data for the genus *Lactobacillus* are as yet very limited. Dias & Weimer (1998) reported the presence of CBL and methionine aminotransferase activities in some strains of lactobacilli. Moreover, a cystathionine γ -lyase was purified from *Lactobacillus fermentum* DT 41, a strain contained in the natural starter used for Parmesan cheese production (Smacchi & Gobetti, 1998). This enzyme was found to be stable under the conditions of cheese ripening and has therefore been thought to contribute to the biosynthesis of volatile sulfur-containing compounds, although L-methionine and L-cysteine were not the optimal substrate for the purified enzyme. Recently, methionine aminotransferase activities have been detected in 30% of *Lactobacillus casei* and 23% of *Lactobacillus plantarum* strains (Amarita *et al.*, 2001).

To our knowledge there are no data available on the catabolism of sulfur-containing amino acids in *Lactobacillus delbrueckii* species. In the present work, 14 strains of *Lb. delbrueckii* subsp. *bulgaricus* or subsp. *lactis* were screened for CBL activity. The corresponding *patC* gene was cloned from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489, sequenced, and its distribution and expression assessed among lactic acid bacteria.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. All *Lactobacillus* strains were grown at 40 °C without shaking in Difco lactobacilli MRS broth (De Man *et al.*, 1960) or in the minimal defined medium described by Poolman & Konings (1988) and modified as follows: 0.008 g tyrosine l⁻¹, 0.4 g cysteine l⁻¹, 0.3 g glutamic acid l⁻¹ and 0.1% (v/v) Tween 80. *Escherichia coli* strains were grown at 37 °C either in Luria-Bertani medium (Miller, 1972) or on minimal medium M63 [0.1 M KH₂PO₄, 15 mM (NH₄)₂SO₄, 9 μ M FeSO₄, 0.8 mM MgSO₄, 3 μ M thiamin chlorhydrate] supplemented with 0.2% (w/v) glucose or glycerol as the carbon source, and 0.01% (w/v) methionine when necessary. Ampicillin, chloramphenicol and erythromycin were added to final concentrations of 100, 20 and 50 μ g ml⁻¹ respectively. Genes under the control of IPTG-responsive promoters were induced for 4 h with 1 mM IPTG at an OD₆₀₀ value of 0.6.

Genomic DNA extraction and Southern-blotting. Genomic DNA of *Lactobacillus* strains was purified using Qiagen Genomic-tip 100/G columns according to the manufacturer's

recommendations (Qiagen). For efficient lysis of the *Lactobacillus* strains, the Qiagen bacterial lysis buffer B1 was supplemented with mutanolysin (10 μ g ml⁻¹). Purified genomic DNA was digested with *Eco*RI. Fragments were separated on an 0.8% (w/v) agarose gel by electrophoresis and transferred onto a nylon filter (Positive membrane, Qbiogene-Quantum Appligene) by the Southern transfer method (Southern, 1975). The DNA probe (for example, the 569 bp *Nco*I–*Sac*I fragment shown in Fig. 1) was directly labelled with horseradish peroxidase using the ECL direct nucleic acid labelling system according to the manufacturer's instructions (Amersham Biosciences). Detection of specific hybridization was done by chemiluminescence using the ECL detection system.

Construction of plasmids. Plasmids constructed in this study are listed in Table 1. A fragment including the gene of interest was amplified from genomic DNA of *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 and *Lb. delbrueckii* subsp. *lactis* ATCC 21815 by PCR with two pairs of primers based on the sequence of the NCDO 1489 *patC* gene (Fig. 1): 5'-GC-GGAATTCGCATGCGGAGAGAGAACG-3' (pma12) and 5'-GCGGGATCCGCATGCCGTTCTACTGGC-3' (pma11), or 5'-GCGGCATGCCAGAAAAGCAATAT-3' (pma14) and 5'-GCAAGCTTCGTTCTAC TGGC-3' (pma15) contain an *Eco*RI, *Bam*HI, *Sph*I and *Hind*III site, respectively (underlined). The 1.4 kb PCR fragments obtained from strains NCDO 1489 and ATCC 21815 were digested with *Eco*RI and *Bam*HI, and then were cloned either in pJDC9 (Chen *et al.*, 1987) or pCR-Script CamSK(+), resulting in plasmids pDL19 and pDL43. The 1.2 kb PCR fragment obtained from NCDO 1489 genomic DNA was cloned as a *Sph*I–*Hind*III fragment into pQE-32 (Qiagen), resulting in plasmid pDL22. Subsequently, the 832 bp *Bgl*II–*Sac*I fragment from pDL22 was replaced by the corresponding pDL43 fragment, resulting in plasmid pDL44, which encodes a hybrid PatC protein. Plasmids pDL22 and pDL44 were used for tagging the protein with six consecutive histidine residues for simple purification using nickel resin.

RNA preparation and RT-PCR. RNA was purified from 1 ml of a fresh bacterial culture grown in MRS medium and harvested at OD₆₀₀ value of 1.0. Then, bacterial cells were treated with the Rneasy Mini kit (Qiagen) according to a modified procedure. Cells were lysed with TE buffer containing 5 mg lysozyme ml⁻¹ (45 min at 37 °C). Genomic DNA was eliminated by the action of the Rnase-free Dnase I (Qiagen) during RNA purification on the Rneasy Mini column (40 min at room temperature). RNA preparations were analysed by agarose gel electrophoresis and RNA concentrations were determined by absorbance at 260 nm.

cDNAs from *patC* and *prtB* mRNA were generated by RT-PCR using the Qiagen OneStep RT-PCR kit according to the supplier's instructions. Two amounts of RNA (0.15 or 1.0 μ g) were used for each RT-PCR assay. Primers specific to *prtB* or *patC* genes from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 were used for amplification (Fig. 1). Primers PRTBsens1 (5'-GACAACCCACTCACTGGACAAG-3') and PRTBrev1 (5'-CTCTTGATCGTAAGCCGGGC-3'), primers PRTBsens2 (5'-TTGACGAAGAAAGATCAGGCCG-3') and PATCrev1 (5'-AACGCCGCCAAGTTGAAGG-3') generated a 504 bp or a 3326 bp amplicon, respectively. cDNA of *patC* (674 bp) was generated with PATCsens1 (5'-GGGCAATTCATCAAGTGGG-3') and PATCrev1 primers. Before any RT-PCR assays, all primers were controlled by direct PCR using a ready-to-use PCR mixture of Gibco-BRL (Invitrogen) and genomic DNAs of four studied strains as templates. In order to check DNA contamination of RNA preparations,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference†
<i>Lb. delbrueckii</i>		
subsp. <i>bulgaricus</i>		
NCDO 1489	Wild-type	NCDO
CNRZ 397	Wild-type	CNRZ
CNRZ 1187	Wild-type	CNRZ
CNRZ 416	Wild-type	CNRZ
LFi5	Wild-type	Germond <i>et al.</i> (1995)
LFi5.H7	Spontaneous <i>lacZ</i> deletion mutant of LFi5	Germond <i>et al.</i> (1995)
LB4	Wild-type	P. Ritzenthaler
LT1	Wild-type	P. Ritzenthaler
NZDRI 5005	Wild-type	NZDRI
<i>Lb. delbrueckii</i>		
subsp. <i>lactis</i>		
CNRZ 250	Wild-type	CNRZ
CNRZ 245	Wild-type	CNRZ
ATCC 21815	Wild-type	ATCC
LL44	Wild-type	NCC
N141	Wild-type	NCC
<i>E. coli</i>		
NM522	F' <i>lacI^a Δ(lacZ)M15 proA⁺B⁺/supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (r_k⁻ m_k⁻ McrBC⁻)</i>	Pharmacia
CAG18475	MG1655 <i>metC162::Tn10</i>	Singer <i>et al.</i> (1989)
Plasmids		
pDL19	pJDC9 carrying <i>patC</i> from NCDO 1489 on a 1.4 kb <i>EcoRI</i> – <i>Bam</i> HI fragment; Er ^r	This study
pDL22	pQE-32 carrying <i>patC</i> from NCDO 1489 on a 1.2 kb <i>Sph</i> I– <i>Hind</i> III fragment; Ap ^r	This study
pDL43	pCR-ScriptCamSK(+) carrying <i>patC</i> from ATCC 21815 on a 1.4 kb <i>EcoRI</i> – <i>Bam</i> HI fragment; Cm ^r	This study
pDL44	pDL22 derivative encoding the hybrid PatC _{NCDO 1489/ATCC 21815}	This study

* Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Er, erythromycin.

† NCDO, National Collection of Dairy Organisms (Food Research Institute, Reading, UK); CNRZ, Centre National de Recherches Zootechniques (Jouy en Josas, France). P. Ritzenthaler (Laboratoire de Microbiologie et de Génétique Moléculaire du CNRS, Toulouse, France); NZDRI, New Zealand Dairy Research Institute (Palmerston North, New Zealand; gift of F. G. Martley); ATCC, American Type Culture Collection (Manassas, VA, USA). NCC, Nestlé Culture Collection (Lausanne, Switzerland).

Fig. 1. Genetic organization of the *prtB*–*patC* locus of *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489. Locations of primers used for gene cloning and RT-PCR experiments are shown.

direct PCR was also carried out with the same primers and Gibco-BRL mixture.

Total cellular extracts for enzyme assays. Cells from 300 ml of an overnight culture of *Lactobacillus* strains grown in Difco MRS broth were harvested, washed with 100 mM Tris/HCl pH 8.2 and then resuspended in 3 ml of the same buffer. The cells were afterwards disrupted at 6 °C by three passages through an Aminco French pressure cell at 20000 p.s.i. (138 MPa). Intact cells and cell debris were removed by centrifugation at 12000 *g* for 30 min at 6 °C. The supernatant was collected and protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as the

standard. The extracts were used for enzyme assays as described below and stored at –20 °C.

Detection of CBL activity by *in situ* staining. CBL activity was monitored in PAGE gels with either L-cystine or L-cysteine as the substrate. Crude extracts (0.3 mg) supplemented with SDS-free sample buffer [5 × buffer is composed of 150 mM Tris/HCl pH 6.8, 30 % (v/v) glycerol, 0.005 % (w/v) bromophenol blue] were loaded on the polyacrylamide gel. A discontinuous system consisting of stacking and separation gels was used to separate proteins. The lower separation gel consisted of 12 % (w/v) polyacrylamide in 150 mM Tris/HCl pH 8.8 with 0.1 % (w/v) ammonium persulfate and 0.1 %

(w/v) tetramethylethylenediamine (TEMED). The stacking gel was composed of 4.8% (w/v) polyacrylamide in 200 mM Tris/HCl pH 6.8 with polymerization reagents at the same concentration as above. The electrophoresis buffer contained 192 mM glycine, 25 mM Tris at pH 8.3. After electrophoresis, the polyacrylamide gel was incubated in 100 mM Tris/HCl buffer pH 8.2 prewarmed to 37 °C and supplemented with 10 mM L-cysteine or L-cystine, 0.5 mM Pb(NO₃)₂ and 0.4 mM PLP.

CBL assay and kinetic constant determination. The assay used for determination of kinetic parameters was based on the formation of free thiol groups by spontaneous disulfide interchange with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] or DTNB according to the method described by Uren (1987). Assay mixtures contained 0.2 mM DTNB, 0.1 mM PLP, various substrate concentrations (L-cystine or L-cystathionine dissolved in water and 0.04 M HCl, respectively) ranging from 0.5 to 4 mM and purified His-tagged PatC_{NCDO1489} in a final volume of 1 ml Tris/HCl 100 mM pH 8. The increase in absorbance at 412 nm was measured in a Uvikon 930 spectrophotometer at 37 °C; it was recorded at 0.5 min intervals for 10 min. A molar absorption coefficient for the aryl mercaptide of 13 200 l mol⁻¹ cm⁻¹ was used to calculate the enzyme activity. One enzyme unit represents the formation of 1 µmol aryl mercaptan min⁻¹. The Lineweaver-Burk plot (1/v against 1/s) was employed to calculate K_m and V_{max} values. The values reported represent a mean of three independent experiments ± SD.

RESULTS AND DISCUSSION

Isolation and sequence analysis of the *patC* gene from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489

The DNA region located downstream from the *prtB* gene encoding the cell-surface proteinase from strain NCDO 1489 (Gilbert *et al.*, 1996) was isolated by inverse PCR, and the DNA sequence was determined on both strands (Fig. 1). A computer-assisted search for protein-coding regions revealed one ORF of 1170 bp which encodes a protein of 390 amino acids with a predicted molecular mass of 43.9 kDa. This ORF was preceded by a potential ribosome-binding site (GAGG) ending 8 bp upstream of the ATG start codon and by several sequences showing high homology to the *Lb. delbrueckii* consensus promoter (Matern *et al.*, 1994). The putative -35 (TGATTT) and -10 (TTACTT) boxes with a spacing of 18 nucleotides and ending 39 bp upstream of the ribosome-binding site seemed to fit better with the consensus promoter for two major reasons: (i) the nucleotides TGC were found upstream of the -10 region; (ii) a potential upstream (UP)-element, characterized by oligo-A and -T stretches (A and T content of 78.2 mol%), was detected 4 nucleotides upstream of the -35 region. This UP-element was shown to stimulate transcription in bacteria and has been found upstream of numerous promoters of *Lb. delbrueckii* genes (Matern *et al.*, 1994). The GC content of this ORF is 52 mol%, which is in good agreement with the characteristic mol% GC content of *Lb. delbrueckii* subsp. *bulgaricus* (Kilpper-Balz *et al.*, 1982).

A search for protein homology revealed significant similarity of the deduced amino acid sequence to the potential amino transferase (PatB) protein (37.3%

Fig. 2. Alignment of the PatC amino acid sequence from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 (PatC_{NCDO1489}) with PatB from *Bacillus subtilis* (Swissprot Q08432) and MalY of *E. coli* (P23256). Boxes indicate identical amino acid residues over the three proteins; conserved amino acids with either PatB or MalY are indicated by a dash above the sequence. Amino acids of PatC from ATCC 21815 and CNRZ 250 which differ from NCDO 1489 are indicated underneath the PatC_{NCDO1489} sequence: white letters on a black background and in bold indicate substitutions in PatC_{ATCC21815} and PatC_{CNRZ250} respectively. Letters shown in bold and underlined indicate substitutions that were found in PatC_{ATCC21815} as well as in PatC_{CNRZ250}. The potential binding site of PLP is shown by a black triangle; amino acids that might be involved in PLP stabilization are indicated by a + symbol (Clausen *et al.*, 2000). Other residues essential for the MalY CBL activity are shown by an asterisk. The lines numbered I through III correspond to regions described by Clausen *et al.* (2000) as being involved in the interaction between MalY and MalT.

identity) of *Bacillus subtilis* and the MalY protein (31.4% identity) of *E. coli* (Fig. 2). Whereas the biological function of PatB is still unknown (Trach & Hoch, 1993), MalY has been described as a pleiotropic protein acting as a PLP-dependent CBL and as a repressor of the maltose regulon by interaction with MalT, the central transcription activator of the maltose regulon (Zdych *et al.*, 1995; Clausen *et al.*, 2000).

Considering the significant identity of the deduced amino acid sequence with PatB from *B. subtilis* (Fig. 2), the ORF located immediately downstream of *prtB* gene from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 was named *patC*. The DNA sequence of the *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 *patC* gene has been assigned GenBank number AF423071.

PatC and CBL activity *in vivo*

Amino acid sequence alignments (Fig. 2) showed that the Lys-233 residue by which the cofactor PLP is covalently attached to MalY, as well as 7 out of 11 residues which had been previously reported to stabilize the cofactor, were conserved in PatC (Clausen *et al.*, 2000). Moreover, the Tyr-121 and Asp-201 residues, essential for the MalY CBL activity, were also conserved in PatC. All these data suggested that PatC might represent a PLP-dependent enzyme with CBL activity.

To test our hypothesis, plasmid pDL19 (harbouring the 1.4 kb PCR fragment encompassing *patC* from NCDO 1489 and its 217 bp upstream region) was constructed and introduced into *E. coli* strain CAG18475, which contains a mutation in the CBL-encoding gene (*metC*) essential in the methionine biosynthetic pathway (Singer *et al.*, 1989). *E. coli* strain CAG18475 harbouring plasmid pDL19 was able to grow on minimal medium M63 supplemented with glucose or glycerol as the carbon source, whereas the same strain carrying the parental vector pJDC9 still required methionine to grow on the same medium. Thus, we clearly demonstrated that the overproduction of PatC, as with MalY (Zydh *et al.*, 1995), complements the methionine requirement of a *metC* mutant and that PatC functions *in vivo* as a CBL. Moreover, we confirmed that the *patC* gene of strain NCDO 1489 is expressed under its own promoter since the fragment cloned in pDL19 is flanked by efficient transcriptional terminators which prevent any interference by vector promoters (Chen & Morrison, 1987).

Although PatC and MalY share some features, they differ in their number of subunits and their capacity to repress the maltose regulon of *E. coli*. The native enzyme MalY consists of two identical subunits (Clausen *et al.*, 2000), whereas PatC from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 is apparently a homotetramer (data not shown) as for CBL from numerous bacteria (Alting *et al.*, 1995; Belfaiza *et al.*, 1986). In contrast to MalY, PatC overproduction did not repress the expression of a *malK'*-*lacZ* fusion (data not shown). So, PatC is not able to control the regulation of the maltose regulon (*malT*-dependent genes). This is not surprising, since most amino acid residues involved in the interaction with the transcriptional activator MalT are not conserved in PatC, as shown in Fig. 2 (Clausen *et al.*, 2000).

Purification of PatC and biochemical features

In order to produce large amounts of PatC, the *patC* gene from NCDO 1489 was amplified from genomic DNA using primers pmal4 and pmal5 (Fig. 1) and

Fig. 3. SDS-PAGE (a) and non-denaturing PAGE (b) analysis of purified His-tagged PatC proteins with Coomassie blue staining and *in situ* staining for CBL activity. Lanes: 1, purified hybrid PatC_{NCDO 1489/ATCC 21815}; 2, purified PatC_{NCDO 1489}; M, molecular mass markers (Gibco-BRL, BenchMark prestained protein ladder). The PatC bands are arrowed.

subsequently cloned in-frame with a His₆-tag coding sequence (pDL22). Moreover, this hybrid *patC* gene was under control of the IPTG-inducible T5 coliphage promoter. The His-tagged PatC protein was purified under native conditions by affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA) resin and its purity was confirmed by SDS-PAGE. Indeed, a single band running at the expected size of 45 kDa was observed following staining with Coomassie blue as shown in Fig. 3(a, lane 2).

CBL activity could easily be monitored in non-denaturing gels using either L-cysteine or L-cystine as the substrate; both compounds have a β -S bond. Hydrogen sulfide resulting from L-cysteine or L-cystine cleavage precipitated with Pb(NO₃)₂ to an insoluble PbS product forming brown to black bands on the polyacrylamide gel. Electrophoresis under non-denaturing conditions of purified His-tagged PatC revealed a single dark brown band (Fig. 3b, lane 2). This suggests that the 13 additional amino acids at the N-terminus of PatC probably do not interfere with enzyme activity. Suppression of the methionine auxotrophy in a *metC* mutant harbouring pDL22 corroborates our hypothesis.

Previous results (Aubel *et al.*, 2002) have shown that like most CBLs (Alting *et al.*, 1995; Smacchi & Gobetti, 1998; Bruinenberg *et al.*, 1997; Gentry-Weeks *et al.*, 1993), PatC is a PLP-dependent enzyme that is sensitive to hydroxylamine. PatC is also sensitive to the thiol-reactive agent iodoacetic acid and, in this respect, is similar to the CBL of *Bordetella avium* (Gentry-Weeks *et al.*, 1993). Nevertheless, the two enzymes differ in their thermostability (Gentry-Weeks *et al.*, 1993; Aubel *et al.*, 2002) and substrate affinity. Indeed under the conditions used (see Methods), the PatC enzyme exhibited slightly higher affinity for L-cystine (K_m 1.88 ± 0.37 mM; V_{max} 48.69 ± 0.95 U mg⁻¹) than for L-cystathionine (K_m 5.13 ± 0.42 mM; V_{max} 13.17 ± 1.01 U mg⁻¹). Both of these K_m values were higher than those reported for the MetC enzyme of *B. avium* (K_m

Fig. 4. Hybridization of genomic DNA from *Lb. delbrueckii* subsp. *bulgaricus* (a) and *Lb. delbrueckii* subsp. *lactis* (b) strains with the *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 *patC* gene. An internal 569 bp DNA fragment of the *patC* gene was hybridized to *Eco*RI-digested genomic DNA (a) of NCDO 1489 (lane 1), Lfi5.H7 (lane 2), LB4 (lane 3), CNRZ 1187 (lane 4), CNRZ 416 (lane 5), Lfi5 (lane 6), NZDRI 5005 (lane 7), CNRZ 397 (lane 8) and LT1 (lane 9), or (b) of NCDO 1489 (lane 1), *Lb. delbrueckii* subsp. *lactis* CNRZ 250 (lane 2), CNRZ 245 (lane 3), LL44 (lane 4), N141 (lane 5) and ATCC 21815 (lane 6). Arrows indicate the sizes of the hybridizing fragments.

0.070–0.077 mM for L-cystathionine and L-cystine, respectively; Gentry-Weeks *et al.*, 1993) and *E. coli* (K_m 0.04–0.25 mM; Dwivedi *et al.*, 1982) or *Salmonella* (K_m 0.22–0.80 mM; Guggenheim, 1971). On the other hand, the affinity of PatC and MalY of *E. coli* (K_m 1.70 ± 0.10 mM; Zdych *et al.*, 1995) for L-cystine was quite similar.

Distribution of the *patC* gene among lactic acid bacteria

An ORF with 97.7 and 98.4% identity to *patC* was detected downstream of the *prtB* genes of *Lb. delbrueckii* subsp. *lactis* CNRZ 250 and ATCC 21815, respectively, which suggested that the locus encompassing both genes was common to most *Lb. delbrueckii* strains. In order to determine whether the *patC* gene was specific to *Lb. delbrueckii* species or widely distributed in lactic acid bacteria, Southern hybridizations were performed. The *patC* probe hybridized with a single DNA fragment in all eight *Lb. delbrueckii* subsp. *bulgaricus* and all five subsp. *lactis* strains studied, as shown in Fig. 4(a, b). However, under the conditions used, no hybridizing

Table 2. Relationships between CBL activity and methionine auxotrophy in *Lb. delbrueckii* strains

Strain	PatC activity*	Methionine auxotrophy†
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i>		
NCDO 1489	+	–
Lfi5.H7	+	–
LB4	+	–
CNRZ 1187	+	–
CNRZ 416	+	–
Lfi5	+	–
NZDRI 50005	+	–
CNRZ 397	–	+
LT1	–	+
<i>Lb. delbrueckii</i> subsp. <i>lactis</i>		
CNRZ 250	+	–
CNRZ 245	–	+
ATCC 21815	–	+
LL44	–	+
N141	–	+

* PatC activity was detected with the *in situ* enzymic test on native PAGE gels (see Methods).

† Methionine auxotrophy was determined after bacterial growth on minimal growth medium without methionine.

band was detected in the genome of *Lactobacillus helveticus* (four strains), *Lb. casei* (one strain) or *Lc. lactis* (three strains) (data not shown). The absence of hybridization with the *Lc. lactis* genome is not surprising, because the identity between PatC and MetC from *Lc. lactis* (Fernandez *et al.*, 2000) is very low (18.3%). Our results suggest that the *patC* gene is only present in *Lb. delbrueckii* species.

CBL biosynthesis and methionine auxotrophy among *Lb. delbrueckii* species

As shown above, sequences homologous to *patC* of NCDO 1489 have been detected in 13 *Lb. delbrueckii* subsp. *bulgaricus* or subsp. *lactis* strains. However, CBL activity could only be detected in cellular extracts of six (CBL⁺) out of eight *Lb. delbrueckii* subsp. *bulgaricus* strains and in a single strain of *Lb. delbrueckii* subsp. *lactis* (CNRZ 250) with the *in situ* enzymic test on native PAGE (Aubel *et al.*, 2002; Table 2). Enzyme activity was also assayed by determining the formation of free thiol groups (Uren, 1987). The specific activity of the crude extract of NCDO 1489 (control strain) towards the substrate L-cystine was $76 \times 10^{-4} \mu\text{mol min}^{-1} \text{mg}^{-1}$. In contrast, the values were eight- to tenfold weaker for strains ATCC 21815 and CNRZ 397, previously described as CBL[–] strains (Aubel *et al.*, 2002). Therefore, the *in situ* enzymic test and the quantitative CBL assay yielded similar results. Whereas CBL⁺ strains were able to grow in a minimal defined medium without meth-

ionine, all CBL⁻ strains were auxotrophic for methionine (Table 2). These results suggest that PatC is probably involved in the methionine biosynthesis pathway of *Lb. delbrueckii* strains.

The mechanism(s) that might be involved in the lack of CBL activity in some strains have been investigated and, therefore, the *patC* genes of two other *Lb. delbrueckii* strains, CNRZ 250 (CBL⁺) and ATCC 21815 (CBL⁻), were sequenced (data not shown). The PatC amino acid sequence from NCDO 1489 (PatC_{NCDO 1489}) differs from PatC_{ATCC 21815} and PatC_{CNRZ 250} at 7 positions (Fig. 2). The substitutions in positions 23, 46, 257 and 327 do not interfere with the enzyme activity since they were found in PatC_{ATCC 21815} (CBL⁻) as well as PatC_{CNRZ 250} (CBL⁺). On the other hand the three substitutions Val-100, Leu-158 and Lys-186, which are only detected in PatC_{ATCC 21815} (CBL⁻), may account for the lack of CBL activity in this strain. In order to test this hypothesis, we constructed a hybrid PatC_{NCDO 1489}/ATCC 21815 protein (plasmid pDL44); as a consequence the Met-100, Pro-158 and Glu-186 of PatC_{NCDO 1489} were respectively substituted by Val, Leu and Lys. The purified hybrid PatC displayed similar enzyme activity to PatC_{NCDO 1489}, as shown in Fig. 3(b), suggesting that the lack of CBL activity in strain ATCC 21815 was not due to these amino acid substitutions. Consequently, PatC_{ATCC 21815} is enzymically active; this was confirmed by the observation that the overexpression of PatC_{ATCC 21815} complemented the methionine auxotrophy of the *E. coli metC* mutant. Therefore, it could be supposed that the transcription of the *patC* gene may be altered in CBL⁻ strains of lactobacilli compared to CBL⁺ strains. To check our hypothesis, total RNA was extracted from two CBL⁺ strains (NCDO 1489 and CNRZ 250) and two CBL⁻ strains (CNRZ 397 and ATCC 21815) belonging to the *bulgaricus* or *lactis* subspecies, respectively. No cDNA was obtained by RT-PCR using PRTBsens2 and PATCrev1 primers (Fig. 1). These results indicate that *prtB* and *patC* do not form an operon in *Lb. delbrueckii*. This conclusion is in agreement with the presence of a CBL activity in LFi5.H7, a spontaneous *lacZ-prtB* deletion mutant (Germond *et al.*, 1995). Moreover, no ORF was detected immediately downstream of the *patC* gene. Thus, expression of *patC* is quite different from that of the *metC* gene that encodes the CBL from *Lc. lactis*, which is co-transcribed with the downstream *cysK* gene encoding a putative cysteine synthase (Fernandez *et al.*, 2000). On the other hand, *patC* and *prtB* cDNAs of the expected size were generated by RT-PCR from all RNA extracts studied (Fig. 5), demonstrating that the *patC* gene was transcribed in CBL⁻ as well as in CBL⁺ strains. All these data suggested that unknown post-transcriptional mechanisms account for the differences in CBL activities.

In conclusion, like most lactic acid bacteria, *Lb. delbrueckii* synthesizes a CBL, which we have named PatC. The sequence and biochemical properties of this enzyme are different from those of other published CBLs (MetC) involved in methionine metabolism. Among *Lb. delbrueckii* species, the biosynthesis level of PatC is variable

Fig. 5. Amplification of cDNAs of *patC* and *prtB* from CBL⁺ or CBL⁻ strains of *Lb. delbrueckii*. RT-PCR was carried out with primers PATCsens1 and PATCrev1 (lanes 2–5) or primers PRTBsens1 and PRTBrev1 (lanes 6–9) using 0.15 µg RNA from *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489 (lanes 2 and 6) or CNRZ 397 (lanes 3 and 7), and *Lb. delbrueckii* subsp. *lactis* CNRZ 250 (lanes 4 and 8) or ATCC 21815 (lanes 5 and 9). Product length was controlled by reference to components of 1 kb ladder (0.8 µg, Gibco-BRL, Invitrogen) (lane 1).

and strain dependent. Further studies are necessary to elucidate mechanisms controlling PatC biosynthesis. Methionine is a precursor of several aroma compounds, and a screening of CBL⁺ strains for cheesemaking could enhance the development of flavours.

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