***Lactococcus lactis* LM0230 contains a single aminotransferase involved in aspartate biosynthesis, which is essential for growth in milk**

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Amino acid aminotransferases (ATases), which catalyse the last biosynthetic step of many amino acids, may have important physiological functions in *Lactococcus lactis* during growth in milk. In this study, the aspartate ATase gene (*aspC*) from *L. lactis* LM0230 was cloned by complementation into *Escherichia coli* DL39. One chromosomal fragment putatively encoding *aspC* was partially sequenced. A 1179 bp ORF was identified which could encode for a 393 aa, 43.2 kDa protein. The deduced amino acid sequence had high identity to other *AspC* sequences in GenBank and is a member of the $I\gamma$ family of ATases. Substrate-specificity studies suggested that the lactococcal *AspC* has ATase activity only with aspartic acid (Asp). An internal deletion was introduced into the *L. lactis* chromosomal copy of *aspC* by homologous recombination. The wild-type and mutant strain grew similarly in defined media containing all 20 amino acids and did not grow in minimal media unless supplemented with asparagine (Asn). The mutant strain was also unable to grow in or significantly acidify milk unless supplemented with Asp or Asn. These results suggest that only one lactococcal ATase is involved in the conversion of oxaloacetate to Asp, and Asp biosynthesis is required for the growth of *L. lactis* LM0230 in milk.

Keywords: *Lactococcus lactis*, lactic acid bacteria, aspartate aminotransferase, amino acid biosynthesis, aspartate biosynthesis

INTRODUCTION

Lactococcus lactis is widely used by the dairy industry for the manufacture of fermented milk products. The primary role of *L. lactis* during dairy fermentations is the production of lactic acid from the milk sugar lactose. Additionally, in bacterial ripened cheese varieties where the product may be aged for several months, bacterial enzymes are important for the generation of compounds involved in cheese flavour development (Urbach, 1995).

Strains of *L. lactis* used by the dairy industry are

auxotrophic for a number of amino acids. The specific amino acid requirements are strain specific (Chopin, 1993) but generally include isoleucine, valine, leucine, histidine and methionine. Bovine milk is on average 3.6% protein by weight and the majority of the nitrogen exists as > 11 kDa proteins called caseins (Swaigood, 1985). The non-protein nitrogen (free amino acids and small peptides) fraction of milk is not sufficient to support the growth of lactococci in milk to high cell densities (Juillard *et al.*, 1995a). Therefore, industrially important *L. lactis* strains contain a number of proteolytic and peptidolytic enzymes to liberate amino acids from milk caseins.

The proteolytic pathway of *L. lactis* is a well characterized metabolic system consisting of (at least) a cell-envelope associated proteinase (PrtP) and twelve intracellular peptidases (for a recent review see Christensen *et al.*, 1999). Additionally, a broad specif-

Abbreviations: Ap, ampicillin; ATase, aminotransferase; CFE, cell-free extract; Em, erythromycin; Opp, *Lactococcus lactis* oligopeptide transport system.

The GenBank accession number for the sequence reported in this paper is AF035157

icity oligopeptide transport system (Opp; Tynkynen *et al.*, 1993), two transport systems for di- and tripeptides (Foucaud *et al.*, 1995; Hagting *et al.*, 1994) and at least nine different amino acid transport systems (Poolman, 1993) have been described. Mutants deficient in PrtP and/or Opp have been used to demonstrate that lactococcal strains that are unable to liberate and/or transport casein-derived oligopeptides only achieve 2–10% the final c.f.u. ml⁻¹ in milk compared to PrtP⁺ Opp⁺ strains (Juillard *et al.*, 1995a). This suggests free amino acids and oligopeptides initially present in milk do not contribute significantly to the overall growth of *L. lactis*. *In vitro* data suggests that lactococci can obtain all amino acids through hydrolysis and transport of β -casein-derived oligopeptides (Juillard *et al.*, 1995b). However, more recent *in vivo* studies have shown that lactococci utilize only a limited number of oligopeptides derived from the C-terminal end of β -casein (Kunji *et al.*, 1998).

It is clear that PrtP and Opp are indispensable for growth of lactococci in milk to high cell densities, and that the rate of hydrolysis of caseins by PrtP is growth-rate limiting (Bruinenberg *et al.*, 1992; Juillard *et al.*, 1995a; Helinck *et al.*, 1997). However, the importance of *de novo* amino acid biosynthesis for optimal growth cannot be excluded. In previous work, a *L. lactis* aspartic acid (Asp) auxotroph was isolated that acidified milk at a reduced rate compared to the wild-type strain (Wang *et al.*, 1998, 2000). The molecular basis of the mutation(s) in this strain, which was derived by acriflavine mutagenesis, has not been reported. A mutant strain carrying a plasmid copy of the lactococcal pyruvate carboxylase gene, which is probably involved in Asp biosynthesis, acidified milk faster than the strain lacking this plasmid. However, the acidification rate of this plasmid-carrying mutant strain was still different to that of the parent strain containing the vector alone. Therefore, the construction of isogenic strains is necessary to clarify the importance of Asp biosynthesis during growth of *L. lactis* in milk.

Our laboratory has been studying amino acid amino-transferases (ATases) from *L. lactis*, with particular focus on their role in amino acid catabolism (Gao *et al.*, 1997, 1998; Atilas *et al.*, 2000). ATases also catalyse the last biosynthetic step of many amino acids. Therefore, we are also interested in creating ATase mutants, identifying the amino acid biosynthetic pathways affected and determining whether the diminished ability of *L. lactis* to synthesize specific amino acids affects this organism's growth in milk. This paper describes the cloning and characterization of the aspartate ATase (*aspC*) gene of *L. lactis* LM0230 and the demonstration that strains lacking this gene are unable to synthesize the amino acids Asp or asparagine (Asn). Additionally, the mutant strain was unable to grow in milk, suggesting neither naturally present amino acids and peptides nor oligopeptides liberated from caseins by PrtP and transported by Opp are capable of fulfilling the Asp or Asn requirement of *L. lactis*.

METHODS

Bacterial strains, media and plasmids. The bacteria and plasmids used in this study are listed in Table 1. All cultures were maintained at -80 °C in 6.9% (w/v) non-fat dry milk and 10% (v/v) glycerol. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB; Sambrook *et al.*, 1989) medium at 37 °C with aeration. M9 medium (Gerhardt *et al.*, 1994) was prepared containing all 20 common amino acids at 50 µg ml⁻¹ except where stated. Additionally, M9 medium was supplemented with 20 µg adenine, guanine, uracil and xanthine ml⁻¹. *L. lactis* LM0230 was routinely grown at 30 °C in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose (M17-G) or lactose (M17-L). The medium of Gao *et al.* (1997) containing all 20 amino acids (Asp and Asn both at 0.1 g l⁻¹) was used for defined medium (DM) growth experiments. The minimal medium MS15 (Cocaign-Bousquet *et al.*, 1995), containing the amino acids arginine, glutamate, histidine, isoleucine, leucine, methionine, serine, threonine and valine, was modified as described by Wang *et al.* (1998). Prior to inoculating minimal media, 2 ml aliquots of cells from early stationary phase growth in M17-L were harvested by centrifugation, washed twice with 1 ml 0.85% (w/v) NaCl and resuspended in 1 ml 0.85% NaCl. This cell suspension was used to inoculate MS15 at an initial OD₆₀₀ of 0.10 as determined using a Beckman-Coulter DU-65 spectrophotometer. Skimmed milk for growth studies was purchased from the University of Wisconsin-Madison Dairy Plant and was steamed for 20 min, incubated at 30 °C for 2 h and steamed again for 20 min prior to inoculation. For some experiments, MS15 or milk was supplemented with 0.42 g Asp l⁻¹ or 0.125 g Asn l⁻¹. When growing *Escherichia coli* or *L. lactis* strains containing pTRKL2-based plasmids, erythromycin (Em) (Sigma-Aldrich) was added to media to a final concentration of 500 µg ml⁻¹ or 2 µg ml⁻¹, respectively. To select for *E. coli* strains carrying pMOB and its derivatives, ampicillin (Ap) was added to media to a final concentration of 60 µg ml⁻¹. *L. lactis* strains carrying pJK550 were screened on bromocresol purple/lactose indicator plates (McKay *et al.*, 1973). For experiments using α -complementation, IPTG (Promega) and X-Gal (Life Technologies) were incorporated into agar media at concentrations of 120 µg ml⁻¹ and 20 µg ml⁻¹, respectively.

Molecular biology techniques. Recombinant DNA and plasmid isolation techniques were performed as described by Sambrook *et al.* (1989). Calf intestinal alkaline phosphatase (Promega), T4 DNA ligase (Life Technologies) and restriction enzymes (Life Technologies) were used as recommended by the manufacturer. All DNA primers were synthesized by Life Technologies. *E. coli* transformations were performed with a Gene Pulser following the manufacturer's recommended instructions (Bio-Rad Laboratories). Transformation of *L. lactis* was performed using the procedure of Holo & Nes (1989).

Construction of a genomic library of *L. lactis* LM0230. Chromosomal DNA was isolated from stationary phase M17-G grown *L. lactis* LM0230 by the method of Ausubel *et al.* (1989). DNA was partially digested with *Sau*3AI and fragments were separated on a 1.0% agarose gel. Chromosomal fragments between approximately 6.5 and 9.5 kb were isolated using a GeneCapsule (Geno Technology). Fragments were ligated with alkaline phosphatase treated, *Bam*HI digested pTRKL2. The products of ligation were electroporated into *E. coli* SURE and cells were plated onto LB agar containing Em (LBE). After incubation for 1 d at 37 °C, 2030 white colonies were picked into LBE broth, grown overnight and 125 µl aliquots from each were combined in a centrifuge tube.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Source or reference
<i>E. coli</i>		
SURE	F' <i>proAB lacI^qΔ</i> [M15 Tn10 (Tet ^r)]	Stratagene
DL39	<i>ilvE12 tyrB507 aspC13</i>	LeMaster & Richards (1988); <i>E. coli</i> Genetic Stock Center, Yale University
<i>L. lactis</i>		
LM0230	Plasmid free	Efstathiou & McKay (1976)
JLS400	LM0230 containing a 270 bp internal deletion in <i>aspC</i>	This study
Plasmids		
pTRKL2	Em ^r <i>lacZ</i> , 6.4 kb	O'Sullivan & Klaenhammer (1993)
pMOB	Ap ^r , 1.8 kb	Gold BioTechnology
pG ⁺ host5	Em ^r temperature sensitive lactococcal integration vector, 5.3 kb	Biswas <i>et al.</i> (1993)
pJK550	Lac ⁺ PrtP ⁺ plasmid from <i>L. lactis</i> C2O, 55.0 kb	Yu <i>et al.</i> (1996)
pSUW414	AspC ⁺ pTRKL2 derivative, 15.1 kb	This study
pSUW415	AspC ⁺ pTRKL2 derivative, 14.2 kb	This study
pSUW416	AspC ⁺ pTRKL2 derivative, 16.1 kb	This study
pSUW417	AspC ⁺ pTRKL2 derivative, 13.9 kb	This study
pSUW418	pTRKL2 derivative, 12.0 kb	This study
pSUW419	pMOB derivative containing 2.3 kb <i>SalI</i> fragment of pSUW417	This study
pSUW420	AspC ⁺ pTRKL2 derivative; vector-encoded <i>lacZ</i> promoter located 5' of <i>aspC</i>	This study
pSUW421	AspC ⁺ pTRKL2 derivative; vector-encoded <i>lacZ</i> promoter located 3' of <i>aspC</i>	This study
pSUW422	pTRKL2 derivative encoding Δ <i>aspC</i>	This study
pSUW423	pG ⁺ host5 derivative encoding Δ <i>aspC</i>	This study

* Δ *aspC*, lactococcal *aspC* containing a 270 bp internal deletion; Tet^r, tetracycline resistant.

Plasmid DNA was isolated from the combined cultures by alkaline lysis (Sambrook *et al.*, 1989).

Cloning of *aspC* by complementation in *E. coli* DL39. The plasmid pool created above was electroporated into *E. coli* DL39. Colonies growing on M9 plates lacking Asp were picked into LBE broth and plasmid encoded ATase activities were confirmed by enzyme assays.

DNA sequencing and sequence analysis. DNA sequencing was performed essentially as described by Chen & Steele (1998), using the Tn1000 kit (Gold BioTechnology) to generate nested sets of transposon insertions in pSUW419. Sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer-Applied Biosystems) and a DNA Thermal Cycler 480 (Perkin Elmer-Applied Biosystems). Sequencing reactions were analysed on an ABI 377XL Automated DNA Sequencer at the University of Wisconsin-Madison Biotechnology Center. Additional sequence information was obtained using primer walking to sequence across the *SalI* site of pSUW417. Sequences were analysed using the GCG sequence analysis

package (Genetics Computer Group). Searches for protein sequences similar to the putative AspC sequence were performed using the BLAST network service (Altschul *et al.*, 1997).

Subcloning of the lactococcal *aspC*. PCR subcloning of the *aspC* gene was accomplished using the primers 5'-AAAA-AAAGATCTTCAATAAAGCGAACCAAG-3' (AspC-up) and 5'-ATATAAAGATCTCTAATTCAAAATCAGCCG-3' (AspC-down), and pSUW417 as template DNA (see Fig. 3). The primers were designed with a 6 bp recognition site for *Bgl*II (5'-AGATCT-3') at the 5' end. PCR was performed using the PCR Elongase Kit of Life Technologies. The cycling conditions were: 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min for 30 cycles. The amplified product was digested with *Bgl*II and ligated to alkaline phosphatase treated, *Bgl*II digested pTRKL2. Ligation products were electroporated into *E. coli* SURE and transformants were plated onto LBE supplemented with IPTG and X-Gal. White colonies were picked into LBE broth, plasmids were isolated using alkaline lysis and plasmids carrying the amplified product were identified by restriction digests and enzyme assays.

Preparation of cell-free extracts (CFEs). Overnight cultures of

E. coli (250 ml) were disrupted with glass beads as described by Gao *et al.* (1997), except CFEs were prepared in 100 mM Bistris, pH 6.5.

ATase enzyme assays. When screening *E. coli* derivatives for AspC activity, the Aspartate Aminotransferase UV-test kit of Sigma was used. For quantitative assays with each of the 20 common amino acids, the assay contained in 1.4 ml: CFE (~12 µg protein), 83 mM Bistris (pH 6.8), 6.7 mM amino acid, 54 µM pyridoxal-5-phosphate and 6.7 mM 2-oxoglutarate. The reaction temperature was 30 °C. At 5, 10 and 15 min, 400 µl aliquots of the reaction mixture were withdrawn and added to 200 µl 0.25 M HCl. Zero minute time points were made by adding the components of the enzyme assay directly into 200 µl 0.25 M HCl. After all time points were taken, samples were centrifuged to remove precipitated protein and the glutamate concentration in each stopped reaction was measured using the colorimetric L-glutamic acid assay kit of Roche Biomolecular. Specific activities were calculated as µmol formazan formed min⁻¹ (mg protein)⁻¹, using the extinction coefficient of formazan at 492 nm (19.9 mmol⁻¹ cm⁻¹).

Protein concentrations were determined using the Micro Protein Determination kit of Sigma and BSA as the protein standard.

Construction of *L. lactis* JLS400. An internal deletion was introduced into *aspC* as follows. The lactococcal *aspC* was amplified from pSUW421 using the primers AspC-up and AspC-down. After purification of the amplified product using a QIAquick PCR purification column (Qiagen), the DNA fragment was digested with *Bgl*III, ethanol precipitated and ligated at low concentrations [about 1–5 ng DNA (µl ligation reaction)⁻¹] to favour intramolecular ligation. A 1 µl aliquot of the ligation reaction was used as template DNA in a PCR reaction containing the primers Del1 (5'-P-AAATCCGACC-GCTGTTGCTC-3') and Del2 (5'-P-ATTCTGTTGGATT-TGAAGGC-3') (see Fig. 3). The same PCR cycling conditions as described above for the primers AspC-up and AspC-down were used. The resulting product was purified using a QIAquick column, and was intramolecularly ligated. A third round of PCR and column purification was performed as described above using the primers AspC-up and AspC-down. The resulting DNA fragment was digested with *Bgl*III and ligated into similarly digested pTRKL2. The ligation mixture was transformed into *E. coli* SURE. Restriction analysis was used to identify plasmids carrying the deleted gene (Δ *aspC*) and sequence analysis using primer 5'-TTTGCCCTCTACG-CTTAC-3' was used to screen for plasmids containing the desired 270 bp internal deletion in *aspC*. A plasmid that contained the appropriate deletion was designated pSUW422. The Δ *aspC* fragment was liberated from pSUW422 with *Bgl*III and ligated into similarly digested pG⁺host5 (Biswas *et al.*, 1993), forming pSUW423.

The Δ *aspC* fragment was introduced into the chromosome of *L. lactis* LM0230 using pSUW423 and the double-crossover homologous integration method of Biswas *et al.* (1993). Strains containing the 270 bp deletion in *aspC* were screened for by PCR using the primers AspC-up and AspC-down.

Growth studies in defined medium and skimmed milk. *L. lactis* strains were grown from a 1% inoculum in M17-L containing Em until early stationary phase (9–11 h). A 1 ml aliquot of cells was centrifuged, washed twice with 0.85% NaCl and resuspended in 1 ml 0.85% NaCl. Cells were inoculated into 30 ml DM containing Em or 100 ml steamed milk containing Em to an initial OD₆₀₀ of 0.01 or 0.001,

respectively. Cells were incubated at 30 °C and OD₆₀₀ readings were recorded from DM or clarified milk as described previously (Chen & Steele, 1998). If necessary, cell suspensions after milk clarification or from DM cultures were diluted in 100 mM Bistris, pH 6.5, or water respectively, to obtain an OD₆₀₀ between 0.03 and 0.30. These values were determined to be within the linear range for cell density readings. Readings for pH were determined using an Orion Research model 410A pH meter equipped with a Mettler Toledo Ingold Electrode (Nelson-Jameson). Values for $\mu_{\max}$ are reported as the mean of 4–6 growth experiments $\pm$ SD.

RESULTS

Cloning of *L. lactis* genes which complement the Asp auxotrophy of *E. coli* DL39

The *L. lactis* LM0230 genomic library was electroporated into *E. coli* DL39. This strain is unable to grow in the absence of Asp due to mutations in *aspC* and *tyrB* (LeMaster & Richards, 1988). A total of 33 DL39 derivatives containing chromosomal inserts were isolated on M9 plates lacking Asp. The *Eco*RI digests of the plasmids from the 33 colonies were compared and five patterns were observed (Fig. 1). Enzyme assays indicated four of the five plasmids (pSUW414, pSUW415, pSUW416, pSUW417) encode for aspartate ATase activity. No aspartate ATase activity was detected in CFEs of *E. coli* DL39(pSUW418).

Plasmids pSUW414, pSUW415, pSUW416 and pSUW417 all encode the *L. lactis* LM0230 *aspC*

The construct pSUW417 was chosen for further analysis. A restriction map of the 7.5 kb chromosomal insert was generated (Fig. 2), and deletion and subcloning analyses were used to further localize the gene encoding aspartate

Fig. 1. *Eco*RI restriction endonuclease digestion of five plasmids derived from the *Lactococcus lactis* LM0230 genomic library that complemented the Asp auxotrophy of *E. coli* DL39. DNA fragments were separated on a 1.2% agarose gel after digestion. Lanes: 1, λ $\times$ *Hind*III and ϕ X174 $\times$ *Hae*III molecular mass size standards; 2, pSUW414; 3, pSUW415; 4, pSUW416; 5, pSUW417; 6, pSUW418.

Fig. 2. Restriction endonuclease map of the pSUW417 insert and localization of the *Lactococcus lactis* LM0230 *aspC* gene. The lines marked $\Delta PstI$ and $\Delta SphI$ indicate the fragments deleted in those constructs. The line marked *SalI* indicates the fragment that was subcloned. The AspC phenotype of the constructs is indicated to the right of the lines. P, *PstI*; S, *SalI*; H, *SphI*.

Fig. 3. Schematic representation of *L. lactis* LM0230 *aspC* and the partial *asnS* sequenced from pSUW417. The large arrow designates the putative direction of transcription of *aspC*, as deduced by the nucleotide sequence. Based upon the nucleotide sequence, transcription of *asnS* is in the same direction as *aspC*. The arrows marked Del1 and Del2 indicate the approximate location and direction of the primers used to introduce a deletion into *aspC*. The arrows marked AspC-up and AspC-down indicate the approximate location and direction of the primers used to subclone *aspC*.

ATase activity. Deletion of the *SphI* or *PstI* fragments internal to the chromosomal insert both eliminated AspC activity. Additionally, no AspC activity was

detected when the 2.3 kb *SalI* fragment from the insert was subcloned in pTRKL2 and transformed into *E. coli* DL39. These results suggested the insert-derived *SalI* restriction site was located within *aspC* or a region necessary for *aspC* expression. Therefore, the 2.3 kb *SalI* fragment from pSUW417 was subcloned into pMOB forming pSUW419 and this insert was partially sequenced. Comparison of translated sequences from ORFs identified on this fragment with AspC sequences in GenBank suggested the C-terminal 264 aa of the lactococcal AspC were encoded on pSUW419. Therefore, primer walking was used to sequence the remaining part of *aspC* using pSUW417 as template DNA.

Double-stranded sequence data were obtained for a 1.89 kb region of pSUW417. An ORF of 1179 bp which could encode a 393 aa, 43.2 kDa protein was identified. Putative -10 (TAAATA) and -35 (TACAAA) promoter sequences and a putative ribosome-binding site (AGGAAA) were identified 5' of the ORF. No *rho*-independent transcriptional terminator was identified 3' of this ORF by the Terminator program of GCG. BLAST (Altschul *et al.*, 1997) searches found the deduced amino acid sequence for this ORF has identity to AspCs and putative AspCs from other organisms. The highest identities were found with sequences from *Bacillus stearothermophilus* (Bartsch *et al.*, 1996), *Bacillus subtilis* (Sorokin *et al.*, 1996), *Bacillus* sp. strain YM-2 (Sung *et al.*, 1991), *Streptomyces coelicolor* (Redenbach *et al.*, 1996) and *Streptomyces virginiae* (Katayama *et al.*, 1995) (52, 52, 51, 45 and 44% identity to the *L. lactis* ORF over > 382 aa residues, respectively). Additionally, amino acids 236–249 of the *L. lactis* sequence conformed to the PROSITE pattern PS00105 (Hofmann *et al.*, 1999) defining an ATase class I pyridoxal phosphate attachment site. Using this defined motif, Lys₂₃₉ was identified as the attachment site for pyridoxal phosphate, an essential cofactor for ATase activity.

Downstream of *aspC*, a partial ORF was identified (Fig. 3). A BLAST search of the deduced amino acid sequence

Table 2. Growth characteristics of *L. lactis* LM0230 and JLS400 derivatives in minimal media, defined media and skimmed milk

Values for $\mu_{\max}$ are reported as the mean of either 4 (for DM) or 5–6 (for skimmed milk) independent experiments $\pm$ SD. ND, No growth detected. For growth in minimal media, cells were inoculated to an initial OD₆₀₀ of 0.1: +, OD₆₀₀ > 0.6 after 2 d growth at 30 °C; –, OD₆₀₀ < 0.2 after 2 d growth at 30 °C.

	LM0230 (pJK550, pTRKL2)	LM0230 (pJK550, pSUW421)	JLS400 (pJK550, pTRKL2)	JLS400 (pJK550, pSUW421)
$\mu_{\max}$ in DM	0.62 $\pm$ 0.01	0.60 $\pm$ 0.01	0.59 $\pm$ 0.01	0.60 $\pm$ 0.01
$\mu_{\max}$ in skimmed milk	0.40 $\pm$ 0.02	0.39 $\pm$ 0.02	ND	0.38 $\pm$ 0.02
Growth in MS15	+	+	–	+
Growth in MS15 + Asp*	+	+	–	+
Growth in MS15 + Asn*	+	+	+	+

* MS15 minimal media supplemented with 0.42 g Asp l⁻¹ or 0.125 g Asn l⁻¹.

indicated this ORF might encode the N-terminal 75 residues of an asparaginyl-tRNA synthetase.

To confirm the identified ORF encodes the AspC activity detected in *E. coli* DL39(pSUW417), the ORF was amplified by PCR using primers Asp-up and Asp-down (Fig. 3). This fragment was cloned into pTRKL2 in both orientations with respect to the vector *lacZ* promoter, forming pSUW420 and pSUW421. AspC activity was detected in CFEs of both *E. coli* DL39(pSUW420) and DL39(pSUW421).

PCR reactions using the primers Asp-up and Asp-down successfully amplified a 1.5 kb fragment from the plasmid templates pSUW414, pSUW415 and pSUW416. Partial sequencing of these PCR products indicated all three of these plasmids encode *aspC*. No product was amplified when pSUW418 was used as template DNA.

The *L. lactis* LM0230 aspartate ATase has activity with Asp and no detectable activity with any other amino acids

The specific activity of AspC from CFEs of *E. coli* DL39(pSUW420) using Asp and 2-oxoglutarate as amino donor and amino acceptor, respectively, was calculated to be $1.07 \pm 0.06 \mu\text{mol formazan formed min}^{-1} (\text{mg protein})^{-1}$. This value was determined from duplicate assays performed on two independent cultures of *E. coli* DL39(pSUW420). For all of the other common amino acids except for Cys, activities were below the quantifiable limit [$0.06 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]. Assays using Cys as the substrate were inconclusive, as reducing agents interfere with the reaction catalysed by the L-glutamic acid assay kit.

A *L. lactis* ΔaspC derivative requires Asp or Asn supplementation to grow in synthetic media or milk

A *L. lactis* LM0230 derivative containing a 270 bp internal deletion in *aspC* was constructed and designated JLS400. LM0230 and JLS400 were both transformed with pJK550, a plasmid carrying the genes for lactose utilization and PrtP of *L. lactis* C2O (Yu *et al.*, 1996), which are essential for growth to high cell densities in milk. Additionally, the pJK550-containing strains were transformed with either pTRKL2 or pSUW421.

In DM, all four strains [LM0230(pJK550, pTRKL2), LM0230(pJK550, pSUW421), JLS400(pJK550, pTRKL2) and JLS400(pJK550, pSUW421)] entered exponential growth within 4.5–5.0 h after inoculation, produced acid at similar rates and reached a final OD₆₀₀ of 2.2–2.4 (data not shown). Additionally, similar μ_{max} values were calculated for all four strains (Table 2). Growth of LM0230(pJK550, pTRKL2), LM0230(pJK550, pSUW421) and JLS400(pJK550, pSUW421) occurred in MS15 minimal media (Table 2). JLS400(pJK550, pTRKL2) grew in MS15 supplemented with Asn, but did not grow in MS15 or MS15 supplemented with Asp.

Fig. 4. Milk growth (a) and pH (b) curves of *Lactococcus lactis* LM0230(pJK550, pTRKL2) (AspC⁻; ●), JLS400(pJK550, pTRKL2) (AspC⁻; ▲) and JLS400(pJK550, pSUW421) (AspC⁺; □). The plot for LM0230(pJK550, pSUW421) is not shown as it overlapped that of LM0230(pJK550, pTRKL2). Values shown are the means of two samples taken per time point. The graphs are representative data from 5–6 independent growth and pH curves.

LM0230(pJK550, pTRKL2) and LM0230(pJK550, pSUW421) grew similarly in skimmed milk (Fig. 4a and Table 2). However, JLS400(pJK550, pTRKL2) did not grow or produce acid at a detectable rate in milk (Fig. 4). The μ_{max} for JLS400(pJK550, pSUW421) was not statistically different from LM0230(pJK550, pTRKL2) or LM0230(pJK550, pSUW421), although a lag period approximately 1 h longer was observed for this strain compared to the latter two. Three strains of LM0230(pJK550, pSUW421) which were independently constructed all displayed the same lag period when inoculated into milk, suggesting the lag period was not the result of unintended mutations created during strain construction.

Fig. 5. Acidification rates of *Lactococcus lactis* LM0230(pJK550, pTRKL2) (AspC⁺; ●), JLS400(pJK550, pTRKL2) (AspC⁻; ▲), JLS400(pJK550, pSUW421) (AspC⁺; □) and a blank (◆) in milk supplemented with 0.42 g Asp l⁻¹. The plots are representative data from two independent growth experiments. The plot for LM0230(pJK550, pSUW421) is not shown, as it overlapped that of LM0230(pJK550, pTRKL2).

Supplementation of milk with Asp or Asn restored the ability of JLS400(pJK550, pTRKL2) to acidify milk; however, the acidification rate in Asp-supplemented milk was slightly lower than that for LM0230(pJK550, pTRKL2), LM0230(pJK550, pSUW421) or JLS400(pJK550, pSUW421) (Fig. 5). Acidification rates of JLS400(pJK550, pSUW421) in milk supplemented with Asp (Fig. 5) or Asn (data not shown) were indistinguishable from those of LM0230(pJK550, pTRKL2) and LM0230(pJK550, pSUW421).

DISCUSSION

To facilitate the cloning of *aspC*, as well as other genes from *L. lactis* LM0230, a genomic library consisting of 2030 plasmid derivatives was constructed in *E. coli*. Using the formula of Clarke & Carbon (1976), a genomic size of 2.6×10^3 kbp for *L. lactis* LM0230 (Davidson *et al.*, 1996), and a mean chromosomal insert size in pTRKL2 of 7.9 kb (data not shown), we can conclude that the probability of all clonable genes being represented within this library is greater than 99%. This conclusion is supported by the fact that the lactococcal *aspC* was isolated on four different plasmids. One plasmid, designated pSUW418, which did not encode AspC activity, also complemented the Asp auxotrophy of *E. coli* DL39. Preliminary sequence data suggests this plasmid may carry a gene or genes involved in amino acid transport (Dudley & Steele, unpublished data).

AspCs are members of family I of the ATase superfamily (Jensen & Gu, 1996). Dendrograms aligning the sequences of ATases involved in the interconversion of aspartate and oxaloacetate reveal two distinct homology groups defined by Jensen & Gu (1996) as subfamilies I α

and I γ (also defined as subfamilies Ia and Ib respectively by Okamoto *et al.*, 1996). These two families may differ slightly in their catalytic mechanism (Nakai *et al.*, 1999). Subfamily I α includes AspC from *E. coli*, and eukaryote cytosolic and mitochondrial AspCs. Subfamily I γ includes the rest of the known eubacterial and archaeal AspCs. Based upon the results of BLAST searches, the lactococcal AspC is most similar to sequences within the I γ group. The conservation of active site residues suggested to exist only within the I γ subfamily (Nakai *et al.*, 1999), such as Lys₁₀₉ (Lys₁₀₂ in *L. lactis*) and Thr₃₆-Ala₃₇-Gly₃₈ (Thr₃₇-Leu₃₈-Gly₃₉ in *L. lactis*), supports this classification.

No *rho*-independent terminator was identified between the 3' end of *aspC* and the start of the ORF for a putative asparaginyl-tRNA synthetase (*asnS*). The genes for *aspC* and *asnS* in *B. subtilis* are also adjacent on the chromosome (Sorokin *et al.*, 1996), although a transcriptional terminator with $\Delta G = -54.4$ kJ mol⁻¹ (Tinoco *et al.*, 1973) is found immediately 3' of the *aspC* ORF. However, it is unknown whether the lactococcal *aspC* is monocistronic or cotranscribed with *asnS*. The construct pSUW421 was capable of complementing the chromosomal *aspC* deletion in JLS400, suggesting co-transcription of *aspC* with the downstream *asnS* is not essential for expression of *aspC* in *L. lactis*. Further studies on the expression of *aspC* are needed to determine the mode of transcription of this gene.

Bacterial cells typically contain a number of ATases with overlapping substrate specificity (Jensen & Calhoun, 1981). For example, in *E. coli* both AspC and TyrB, an ATase that is preferentially active on aromatic amino acids, catalyse the transamination of oxaloacetate to Asp. However, growth studies in MS15 and milk suggest no other *L. lactis* enzyme can transaminate oxaloacetate *in vivo* efficiently enough to support growth under Asp-limiting conditions. This is consistent with the substrate specificity of the lactococcal aromatic (Gao & Steele, 1998; Yvon *et al.*, 1997) and branched-chain (Atilas *et al.*, 2000; Yvon *et al.*, 2000) ATases, which lack detectable activity with Asp. Asp also appears to be the only amino acid substrate for AspC. Many other subfamily I γ AspC homologues also have relative activities with Asp that are at least 100-fold higher than the activities with other amino acids (Marino *et al.*, 1988; Sung *et al.*, 1990; Xing & Whitman, 1992; Okamoto *et al.*, 1996). However, the inability to detect activity of the lactococcal AspC with amino acids other than Asp may also have been due to the lack of sensitivity of the assay used, which was approximately 6% relative activity to that observed with Asp.

In a previous study a lactococcal strain was isolated after acriflavine mutagenesis which was unable to acidify 11% reconstituted skimmed milk to a pH less than approximately 5.0 in 6 h unless supplemented with Asp (Wang *et al.*, 1998). This strain, designated KB4, produces decreased levels of the protein pyruvate carboxylase (Wang *et al.*, 2000), has approximately 5% the pyruvate carboxylase activity of the wild-type strain

(Wang *et al.*, 1998) and lacks a 12 MDa plasmid present in the parent strain. The exact molecular basis of the mutation or mutations in KB4 has not been reported. As pyruvate carboxylase catalyses the formation of oxaloacetate, the biosynthetic precursor to Asp, this data suggested the growth defect was due to a decreased level of Asp production by KB4. This current study used homologous recombination to construct isogenic strains and demonstrate that a mutation in the *L. lactis* Asp biosynthetic pathway is sufficient to eliminate this strain's ability to grow in milk. During six growth curve replicates, the strain which lacked both the wild-type chromosomal and plasmid copy of *aspC* never acidified milk to more than 0.20 pH units below that of the blank and did not grow at a detectable rate. As oligopeptides liberated from caseins by the lactococcal PrtP serve as the major nitrogen source for *L. lactis* in milk (Juillard *et al.*, 1995a), and *L. lactis* can apparently transport and hydrolyse at least one Asp-containing peptide from β -casein (Kunji *et al.*, 1998), the inability of JLS400(pJK550, pTRKL2) to rapidly grow in or acidify milk suggests *L. lactis* is unable to liberate an adequate level of Asp or Asn from milk caseins to support optimal growth. As Asp is the biosynthetic precursor for Asn, threonine, lysine and methionine, as well as pyrimidine and purine nucleotides, Asp biosynthesis may also be essential for supplying the cell with sufficient levels of these biological compounds. It has previously been shown that *L. lactis* purine auxotrophs are unable to grow in milk (Dickely *et al.*, 1995); however, the necessity of threonine, lysine or pyrimidine biosynthesis is unknown. Asp is probably not diverted towards methionine biosynthesis in dairy lactococci, as these organisms are typically auxotrophic for methionine (Chopin, 1993). Studies using milk supplemented with amino acids and nucleotides will be necessary to determine whether the growth defect of JLS400(pJK550, pTRKL2) is the direct result of the Asp auxotrophy, and/or is an indirect effect on other biosynthetic pathways.

One surprising result from the growth studies was that JLS400(pJK550, pTRKL2) failed to grow in MS15 media supplemented with Asp, but did grow when supplemented with Asn. The most likely explanation is that *L. lactis* LM0230 can convert Asn to Asp via an asparagine synthetase or related enzyme, and transport of free Asn is energetically more favourable than Asp transport. This is supported by previous studies which have shown that lactococci do not readily transport free Asp (Hillier *et al.*, 1978) and that the affinity constant of the Asp transporter is approximately 80-fold higher than the affinity constant of the Asn transporter, for their respective substrates (250 μ M vs 3 μ M, respectively) (Konings *et al.*, 1989). This hypothesis is additionally supported by the fact that JLS400(pJK550, pTRKL2) acidifies Asp-supplemented milk slower than JLS400(pJK550, pSUW421) (Fig. 5), while these two strains acidify Asn-supplemented milk at the same rate (data not shown).

Experiments to clone other ATase genes from *L. lactis*

are ongoing. Construction of single and multiple ATase mutants will be used to further study the physiological role of these enzymes during growth in milk as well as their potential role in the generation of flavour precursors in fermented products.

ACKNOWLEDGEMENTS

This project was supported by the Wisconsin Center for Dairy Research through funding from Dairy Management, Inc., and the College of Agriculture and Life Science at the University of Wisconsin-Madison.

We thank Myrta Atilas and Cedric Mendiola for assistance in construction of the *L. lactis* LM0230 genomic library.

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Received 21 June 2000; revised 20 September 2000; accepted
27 September 2000.