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 Iγ 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 (Swaisgood, 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-
ticity oligopeptide transport system (Opp; Tynkkynen 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., 1995a). 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; Attilas 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 (Cocaing-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 OD₅₆₀ 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 pJK530 were screened on bromocresol purple/lactose indicator plates (McKay et al., 1973). For experiments using β-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 _Sal_III and fragments were separated on a 10 % agarose gel. Chromosomal fragments between approximately 6.5 and 9.5 kb were isolated using a GeneCapule (Geno Technology). Fragments were ligated with alkaline phosphatase treated, BamHI 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.
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 plasmids were isolated using alkaline lysis and plasmids carrying the amplified product 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 (GoldBioTechnology) 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'-AAAAAAAGATCTTCAATAAAGCGAACCAG-3' (AspC-up) and 5'-ATATAAAAGATCTCAATTCAAATCGCCG-3' (AspC-down), and pSUW417 as template DNA (see Fig. 3). The primers were designed with a 6 bp recognition site for BglII (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 BglII and ligated to alkaline phosphatase treated, BglII 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

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**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong> SURE</td>
<td>F' proAB lacZΔM15 Tn10 (Tet&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>DL39</td>
<td>ilvE12 tyrB507 aspC13</td>
<td>LeMaster &amp; Richards (1988); E. coli Genetic Stock Center, Yale University</td>
</tr>
<tr>
<td><strong>L. lactis</strong> LM0230</td>
<td>Plasmid free</td>
<td>Efstatthiou &amp; McKay (1976)</td>
</tr>
<tr>
<td>JLS400</td>
<td>LM0230 containing a 270 bp internal deletion in aspC</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTRKL2</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt; lacZ, 6-4 kb</td>
<td>O'Sullivan &amp; Klaenhammer (1993)</td>
</tr>
<tr>
<td>pMOB</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, 1-8 kb</td>
<td>Gold BioTechnology</td>
</tr>
<tr>
<td>pG&lt;sup&gt;r&lt;/sup&gt;host5</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt; temperature sensitive lactococcal integration vector, 5.3 kb</td>
<td>Biswas et al. (1993)</td>
</tr>
<tr>
<td>pJK550</td>
<td>Lac&lt;sup&gt;c&lt;/sup&gt; Prp&lt;sup&gt;r&lt;/sup&gt; plasmid from L. lactis C2O, 55-0 kb</td>
<td>Yu et al. (1996)</td>
</tr>
<tr>
<td>pSUW414</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative, 15-1 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW415</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative, 14-2 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW416</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative, 16-1 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW417</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative, 13-9 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW418</td>
<td>pTRKL2 derivative, 12-0 kb</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW419</td>
<td>pMOB derivative containing 2.3 kb Sall fragment of pSUW417</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW420</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative; vector-encoded lacZ promoter located 5' of aspC</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW421</td>
<td>AspC&lt;sup&gt;r&lt;/sup&gt; pTRKL2 derivative, vector-encoded lacZ promoter located 3' of aspC</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW422</td>
<td>pTRKL2 derivative encoding ΔaspC</td>
<td>This study</td>
</tr>
<tr>
<td>pSUW423</td>
<td>pG&lt;sup&gt;r&lt;/sup&gt;host5 derivative encoding ΔaspC</td>
<td>This study</td>
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*ΔaspC, lactococcal aspC containing a 270 bp internal deletion; Tet<sup>r</sup>, tetracycline resistant.*
**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 by diluting the components of the enzyme assay directly into 200 μl 0.25 M HCl. After all time points were taken, samples were centrifuged and 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 BglII, 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-GCTTGGCTC-3') and Del2 (5'-P-ATTCTGTGTGATT-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 BglII 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’-TTTGGCCTCTAGGCTTAC-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 AaspC fragment was liberated from pSUW422 with BglII and ligated into similarly digested pG’host5 (Biswas et al., 1993), forming pSUW423.

The AaspC 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 *μ*ₘₐₓ are reported as the mean of 4–6 growth experiments ± 5SD.

**RESULTS**

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

The *L. lactis* LM0230 genomic library was electrooporated 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 EcoRI 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

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**Fig. 1.** EcoRI 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 12% agarose gel after digestion. Lanes: 1, *E. coli* DH5α x HindIII and *X. haeII* molecular mass size standards; 2, pSUW414; 3, pSUW415; 4, pSUW416; 5, pSUW417; 6, pSUW418.
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 rbi-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 virginiiae (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, Lys_39 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

<table>
<thead>
<tr>
<th></th>
<th>LM0230 (pJK550, pTRKL2)</th>
<th>LM0230 (pJK550, pSUW421)</th>
<th>JLS400 (pJK550, pTRKL2)</th>
<th>JLS400 (pJK550, pSUW421)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ_max in DM</td>
<td>0.62 ± 0.01</td>
<td>0.60 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>μ_max in skimmed milk</td>
<td>0.40 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>ND</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>Growth in MS15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in MS15 + Asp*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in MS15 + Asn*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* 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 ± 0·06 μmol formazan formed min⁻¹ (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 μmol min⁻¹ (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 μmax 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.

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 μmax 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.
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 × 10^5 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 Iα and Iγ (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 Iα includes AspC from *E. coli*, and eukaryote cytosolic and mitochondrial AspCs. Subfamily Iγ 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 Iγ group. The conservation of active site residues suggested to exist only within the Iγ subfamily (Nakai et al., 1999), such as Lys109 (Lys102 in *L. lactis*) and Thr30-Ala32-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 ΔG = −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 transaminase 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 (Atiles 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 Iγ 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.

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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).
(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

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