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Original article

Strains of *Lactococcus lactis* with a partial pyrimidine requirement show sensitivity toward aspartic acid

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Abstract – The growth rate of the widely used laboratory strain *Lactococcus lactis* subsp. *cremoris* LM0230 was reduced if aspartic acid were present in the growth medium. The strain LM0230 is a plasmid- and phage-cured derivative of *L. lactis* subsp. *cremoris* C2, the ancestor of the original dairy isolate *L. lactis* subsp. *cremoris* NCDO712. The growth of both C2 and NCDO712 was unaffected by exogenous aspartate. Also, the growth rate of the pyrimidine auxotrophic mutants of *L. lactis* was affected by exogenous aspartate. The maximum observed reduction in the growth rate was ~ 35% when compared with the wild-type strain, and this varied with the concentration of aspartic acid. The observed effect of aspartate could be explained by the accumulation of the toxic pyrimidine de novo pathway intermediate, carbamoyl aspartate. Assays of the pyrimidine biosynthetic enzymes of *L. lactis* LM0230 showed that the partial pyrimidine requirement can be explained by a low specific activity of the pyrimidine biosynthetic enzymes. In conclusion, *L. lactis* LM0230 during the process of plasmid- and prophage-curing has acquired a partial pyrimidine requirement resulting in sensitivity toward aspartic acid.

cell physiology / *Lactococcus lactis* / starter culture / nucleotide metabolism / lactic acid bacteria

摘要 – 偏好嘧啶的 *Lactococcus lactis* 菌株对天冬氨酸敏感。 乳酸乳球菌乳脂亚种（*Lactococcus lactis* subsp. *cremoris*）LM0230 是实验室广泛使用的菌株。当培养基中含天冬氨酸时，该菌株生长速率下降。LM0230 菌株是 *L. lactis* subsp. *cremoris* C2 菌株的质粒和噬菌体治愈的衍生菌株。C2 菌株来自原始的乳品分离菌株 *L. lactis* subsp. *cremoris* NCDO712。*L. lactis* C2 和 NCDO712 菌株的生长均未受到外源天冬氨酸的影响。*L. lactis* 嘧啶营养缺陷型突变菌株的生长速率受外源天冬氨酸的影响，生长速率随着天冬氨酸的浓度变化而变化，与野生型菌株相比，生长速率最高降幅约为 35%。天冬氨酸的增加导致了嘧啶从头生物合成途径中间产物氨基甲酰天冬氨酸积累，进而引起菌株中毒，生长速率下降。*L. lactis* LM0230 嘧啶生物合成相关酶分析表明，嘧啶合成相关酶的特异活性低导致了该菌株对嘧啶的偏好。*L. lactis* LM0230 在质粒和原噬菌体的加工过程中对嘧啶的偏好，导致了菌株对天冬氨酸的敏感性。

细胞生理学 / *Lactococcus lactis* / 发酵剂 / 核苷代谢 / 乳酸菌

Résumé – Des souches de *Lactococcus lactis* ayant un besoin partiel en pyrimidine sont sensibles à l'acide aspartique. Le taux de croissance de la souche *Lactococcus lactis* subsp.

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cremoris LM0230, très utilisée en laboratoire, était réduit si le milieu de croissance contenait de l'acide aspartique. LM0230 est une souche curée de plasmide et de phage issue de *L. lactis* subsp. *cremoris* C2, ces deux souches ayant pour origine commune l'isolat laitier *L. lactis* subsp. *cremoris* NCDO712. La croissance de C2 et de NCDO712 n'était pas affectée par l'aspartate exogène. Par contre, le taux de croissance de mutants auxotrophes pour la pyrimidine était affecté par l'aspartate exogène. Le maximum de réduction du taux de croissance observé était de 35 % comparé à la souche sauvage et variait avec la concentration en acide aspartique. L'effet de l'aspartate observé peut être expliqué par l'accumulation de l'aspartate carbamoyl, intermédiaire toxique dans le métabolisme de la pyrimidine. L'étude des enzymes de biosynthèse de la pyrimidine de *L. lactis* LM0230 a montré que le besoin partiel en pyrimidine peut s'expliquer par une activité spécifique faible de ces enzymes. On peut en conclure que *L. lactis* LM0230 a acquis, au cours du processus de cure de plasmide et de prophage, le besoin partiel en pyrimidine résultant en une sensibilité à l'acide aspartique.

physiologie cellulaire / *Lactococcus lactis* / levain / métabolisme nucléotidique / bactérie lactique

1. INTRODUCTION

In the development of new products in the dairy industry, specific engineering of organisms used for starter cultures is an important tool. If the objective were to obtain strains with specific characteristics, a fundamental knowledge of the central biochemical pathways of the organism of interest is necessary. Such knowledge may be a way to explain the effects observed when altering the medium used to cultivate these strains. One of the most fundamental metabolic pathways is that of nucleotides, nucleosides, and nucleobases. All organisms need nucleotides to synthesize DNA, RNA, and several co-enzymes. This can be achieved either by de novo synthesis or by exploiting nucleotides, nucleosides, and nucleobases salvaged from the surroundings [10].

An understanding of the salvage pathways of pyrimidine compounds is important when dealing with effects on growth arising as a consequence of mutations in the genes of the pyrimidine de novo pathway. These pathways vary between different organisms, but the pathways by which pyrimidine compounds are metabolized in *Lactococcus lactis* are quite similar to those of *Bacillus subtilis* [13, 14]. In contrast to salvage, the de novo synthesis of pyrimidines seems to be universal. This pathway consists of six enzymatic reactions leading to uridylate monophosphate (UMP, Fig. 1) that subsequently is converted into different pyrimidine triphosphates. In lactococci, the organization of the de novo genes do however differ from most of the other gram-positive bacteria characterized so far, as all genes encoding pyrimidine biosynthetic enzymes that are needed to synthesize UMP are not contained in one operon [2, 10, 15, 16].

In growth experiments with the strains of *L. lactis* harboring different mutations in pyrimidine de novo pathway genes, we observed an unexpected effect of exogenous aspartate on the growth rate of these mutants. The different strains used in this study included *L. lactis* subsp. *cremoris* NCDO712 and its various derivatives: C2 (NCDO2031), LM0230, MG1363, MB6 (*pyrDa* and *pyrK*), MB40 (*pyrB*), and MB301 (*pyrF*). The bacterial strains and plasmids used in this study are listed in Table I. Strain LM0230 is a plasmid- and phage-cured derivative of *L. lactis* C2 obtained by nitrosoguanidine and the UV treatment [5]. Strain MG1363 is a

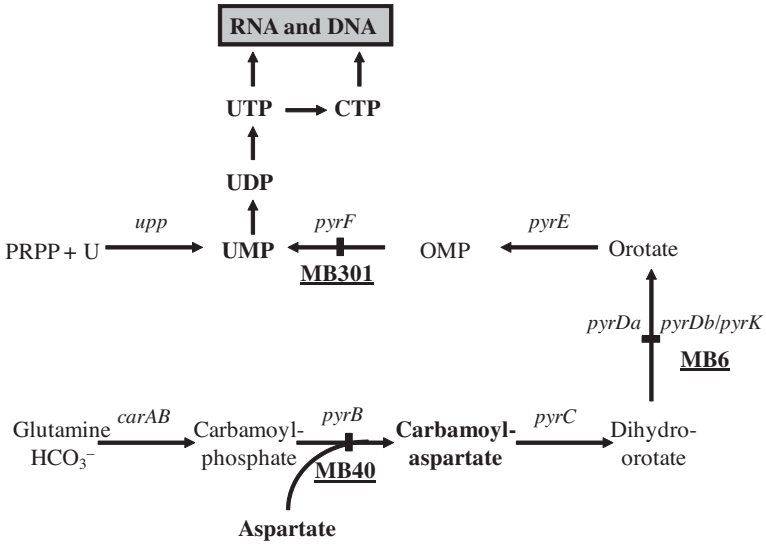

Figure 1. Simplified schematic representation of pyrimidine biosynthesis in *L. lactis* leading to the central pyrimidine compound UMP that subsequently is converted to UTP and CTP. The genes encoding the enzymes catalyzing the individual reactions are shown. U, uracil; UR, uridine; UMP, uridylate (monophosphate); OMP, orotidylate (monophosphate); R-1-P, ribose-1-phosphate; PRPP, 5-phosphoribosyl-1-α-pyrophosphate; *carAB*, carbamoylphosphate synthase; *pyrB*, aspartate transcarbamoylase; *pyrC*, dihydroorotase; *pyrD*, dihydroorotate dehydrogenase; *pyrE*, orotate phosphoribosyltransferase; *pyrF*, OMP decarboxylase; *upp*, uracil phosphoribosyltransferase. MB40, MB6, and MB301 are the three mutant strains lacking the enzymatic activities corresponding to their position.

Table I. Strains and plasmids.

Strains and plasmids	Genotype description and/or construction	Reference or origin
Strains of L. lactis		
MG1363	Plasmid-free derivative of NCDO712	[6]
MB40	MG1363 *pyrB*	This study
MB6	MG1363 *pyrDa/pyrK*	This study
MB301	MG1363 *pyrF*	This study
C2	Wild-type strain	NCDO2031
LM0230	Plasmid-free derivative of C2	[5]
Plasmids		
pG^+ Host4	*ermAM; L. lactis* integration vector	[12]
pIP51	*bla*; *L. lactis pyrK-pyrDb-pyrF*	[1]
pIP61	*bla*, *tet L. lactis pyrDa*	[1]
pIP67	*bla, erm L. lactis ΔpyrDa*	This study
pSH51	*bla; L. lactis ΔpyrF*	This study
pSH54	*bla, ermAM; L. lactis ΔpyrF*	This study

phage- and plasmid-cured derivative of strain NCDO712 obtained by protoplast-induced curing [6]. Both LM0230 and MG1363 have, because of their genetic simplicity, been used intensively as model organisms of lactic acid bacteria. The ancestor of strain C2 (NCDO2031) is NCDO712, and accordingly, strains LM0230 and MG1363 can both be regarded as plasmid- and phage-cured derivatives of strain NCDO712, although obtained by different methods. MB40, MB6, and MB301 are derivatives of MG1363 harboring a *pyrB* mutation, a *pyrDa/pyrK* deletion, and an internal *pyrF* deletion, respectively. The pyrimidine de novo reactions blocked in strains MB6, MB40, and MB301 are shown in Figure 1. The present study reports an investigation into the observation that the growth rate of certain lactococcal strains is reduced in the presence of aspartate. We present evidence that the observed inhibition of growth can be correlated to a pyrimidine requirement and is most likely caused by an accumulation of a pyrimidine de novo pathway intermediate, most probably, carbamoyl aspartate. In accordance with this, an observed effect of aspartate on the growth of *L. lactis* subsp. *cremoris* LM0230 can be explained with at least one mutation in a gene affecting pyrimidine biosynthesis, since the growth of this strain is stimulated by exogenous uracil. Our studies included growth experiments with different strains of *L. lactis* subsp. *cremoris* in a defined medium with varying aspartate concentrations. Furthermore, the levels of pyrimidine de novo enzymes were determined in *L. lactis* subsp. *cremoris* MG1363 and LM0230 in defined medium. The results obtained in this work show that it is relevant to analyze the general physiology of starter cultures obtained after mutagenesis to prevent reduced performance in milk fermentation. Moreover, our findings stress the importance of taking the medium composition into account when working with cells with alterations in their pyrimidine metabolism.

2. MATERIALS AND METHODS

2.1. Growth conditions

Lactococcal cultures were grown on either M17 glucose broth (GM17) [22] or glucose synthetic amino acid (GSA) medium based on MOPS, which contained 7 vitamins, 19 amino acids, and supplied with 10 $g \cdot L^{-1}$ glucose [8]. *Escherichia coli* cultures were grown on Luria-Bertani broth medium and *L. lactis* was cultured at 30 or 37 °C in filled culture flasks without aeration. *E. coli* were grown at 37 °C in batch cultures with vigorous shaking, and to each plate 15 $g \cdot L^{-1}$ of agar was added. As per requirement, the following were added to the different media: NaCl at 10 $g \cdot L^{-1}$, uracil at 20 $\mu g \cdot mL^{-1}$, erythromycin at 1 $\mu g \cdot mL^{-1}$ for lactococci and at 150 $\mu g \cdot mL^{-1}$ for *E. coli*, and ampicillin at 100 $\mu g \cdot mL^{-1}$. All media were obtained from Oxoid (Basinstoke, UK), whereas other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. DNA in vitro manipulation and transformation

Chromosomal lactococcal DNA was prepared as described by Johansen and Kibenich [9]. The methods described by Sambrook et al. were used for general DNA methods in vitro [20]. *L. lactis* was transformed by electroporation [7] and *E. coli* cells were transformed using $CaCl_2$ treatment as described by Sambrook et al. [20].

2.3. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table I. The erythromycin cassette from pIL253 [21] was isolated by digestion with *Hha*I. The resulting fragment was cloned in the *Hinc*II site of

pUC7 [24]. A 1.3-kb *Bam*HI fragment from the resulting construct, containing the erythromycin cassette, was cloned in the *Bam*HI site of pIP61 [1]. The resulting plasmid was linearized with *Nco*I and the 3′-recessed ends were filled in and re-ligated, resulting in *pyrDa*$^{-}$, Ap^{R}, and Em^{R}; the resulting plasmid was named pIP67. In analogy with the procedure described previously for obtaining MB40 from MG1363 using pIP58 [1], MB6 was obtained from MB40 using pIP67 in a double cross-over experiment. The resulting strain carries a deletion of *pyrDb* and a frameshift mutation in *pyrDa*. A 1.2-kb *Eco*RI fragment covering the C-terminal part of *pyrF*, all of *orfC*, and the N-terminal part of *gidB* of the *L. lactis* MG1363 *pyrKDF* operon [2] was obtained by standard PCR with KBJ27 (ATGCAAAATGACCAA) and SLLH1 (GGAATGAATTCATTAATCTGACCGC), the latter containing an *Eco*RI site. This fragment was cloned in the *Eco*RI site of plasmid pIP51 [2]. The orientation of the cloned fragment was verified by PCR using SLLH1 and CLJ2 (GACCTGTTATTGC CCTAG). The resulting plasmid pSH51 carries a 0.7-kb internal deletion of *pyrF*, with about 1.2 kb of flanking sequence on each side. Plasmid pSH51 was digested with *Pst*I and ligated with *Pst*I digested pG^{+}Host4 [12], the resulting plasmid was named pSH54. Competent *L. lactis* MG1363 cells were transformed with plasmid pSH54, unable to replicate in *L. lactis* at 37 °C containing a selectable Erm^{R} marker and a cloned internal deletion of the lactococcal *pyrF*. Transformants were selected and purified on GM17 plates containing erythromycin at 28 °C. These transformants were grown at 37 °C on GM17 plates containing erythromycin, uracil, and 10 g·L^{-1} NaCl to select for the strain in which pSH54 had recombined into the chromosome. After the purification of erythromycin-resistant integrants at 37 °C, the excision of plasmid was facilitated by growing them at 28 °C on plates containing erythromycin and uracil. The excised plasmids were cured from the cells by growing them at 37 °C on plates without erythromycin. The purified mutants were tested for growth on a defined medium (GSA) in the absence and in the presence of uracil. One of the strains shown to have uracil requirement was used for further studies and was named MB301. The mutation in strain MB301 was verified using PCR analysis to be a *pyrF* deletion. Strain MB40 is a derivative of *L. lactis* MG1363 carrying a single *IS*S1 transposon in the middle of the *pyrB* gene, thus resulting in a pyrimidine requirement. It was obtained by plasmid curing of a strain carrying a pG^{+}host4::*IS*S1 in the *pyrB* gene of *L. lactis* MG1363 [17].

2.4. Enzyme assays

Cells were grown exponentially in GSA medium, harvested, washed, and resuspended in 50 mmol·L^{-1} Tris/HCl pH 7.0, 1 mmol·L^{-1} EDTA, and 1 mmol·L^{-1} dithiothreitol, resulting in a 100-fold concentration of the cells. The cells were lysed using a French pressure cell press at 20·10^{6} kg·m^{-2}. Cell debris was removed by centrifugation, and the supernatant was directly used as enzyme source in the assays. The protein concentration was determined according to Lowry et al. [11]. All assays were performed at 30 °C using crude extracts with a final concentration of about 200 g·L^{-1} protein. Specific activities are expressed as units per milligram protein, and one unit is defined as 10^{-6} mol product formed or substrate used per minute. The activities were determined in biological replicates at least two times.

2.4.1. Aspartate transcarbamoylase (PyrB)

Aspartate transcarbamoylase (PyrB) activity was determined at pH 7.0 in the following way: potassium aspartate (50 mmol·L^{-1}),

potassium phosphate (25 mmol·L^{-1}), and enzyme extract were mixed and equilibrated at 30 °C. The assay was started by the addition of carbamoyl phosphate (3 mmol·L^{-1}). Aliquots (150 μL) were extracted between 0 and 20 min, and the formation of carbamoyl aspartate was measured using the colorimetric procedure as described by Prescott and Jones [19], in which 10^{-6} mol carbamoyl aspartate corresponded to an absorption of 18 at 560 nm.

2.4.2. *Dihydroorotase (PyrC)*

Dihydroorotase (PyrC) activity was determined in the backward direction (dihydroorotate to carbamoyl aspartate) essentially as described for the aspartate transcarbamoylase assay with the following alterations. Potassium aspartate and potassium phosphate were substituted by Tris/HCl (100 mmol·L^{-1}) and EDTA (2 mmol·L^{-1}), and the assay was started by adding dihydroorotate (2 mmol·L^{-1}) instead of carbamoyl phosphate [19].

2.4.3. *Dihydroorotate dehydrogenase A (PyrDa)*

Dihydroorotate dehydrogenase A (PyrDa) activity was determined by monitoring orotate formation using a spectrophotometer at 295 nm ($\varepsilon = 3.67 \times 10^3$ mol^{-1}). The reaction mixture contained 0.1 mol·L^{-1} sodium phosphate (pH 7.0), 50 mmol·L^{-1} KCN, and 0.1 mmol·L^{-1} fumarate. After equilibration, the assay was started by the addition of dihydroorotate (0.1 mmol·L^{-1}).

2.4.4. *Dihydroorotate dehydrogenase B (PyrDb)*

Dihydroorotate dehydrogenase B (PyrDb) activity was assayed as dihydroorotate dehydrogenase A, except that fumarate was substituted by 0.1 mmol·L^{-1} NAD^+.

2.4.5. *Orotate phosphoribosyltransferase (PyrE)*

Orotate phosphoribosyltransferase (PyrE) was measured in a buffer at pH 7.5 containing Tris/HCl (20 mmol·L^{-1}), EDTA (2 mmol·L^{-1}), and orotate (300 μmol·L^{-1}). The reaction was followed spectrophotometrically at 295 nm after the addition of 5-phosphoribosyl-1-pyrophosphate. A decrease in absorbancy of 3.67 is equivalent to an increase inorotidylate monophosphate (OMP) of 1 mmol·L^{-1}.

2.4.6. *OMP decarboxylase (PyrF)*

OMP decarboxylase (PyrF) was determined in a buffer at pH 7.5 containing 20 mmol·L^{-1} Tris/HCl and 2 mmol·L^{-1} EDTA. After calibration at 30 °C, the reaction was initiated by the addition of 50 μmol·L^{-1} OMP. The activity was followed using a spectrophotometer at 285 nm. The reduction of the OMP concentration by 1 mmol·L^{-1} corresponds to a decrease in absorbancy of 1.65.

3. RESULTS AND DISCUSSION

3.1. Growth of *L. lactis* LM0230 in different synthetic media

In growth experiments with strain *L. lactis* LM0230, we observed a growth rate reduction of this strain when grown in the defined medium DN (0.23 h^{-1}) formulated by Dickely et al. [4] as compared to growth in the defined GSA medium (0.25 h^{-1}) formulated by Jensen and Hammer [8]. A similar effect could be observed for strains of *L. lactis* harboring mutations in pyrimidine de novo genes. The main difference between the two media is that the DN medium is phosphate buffered and is based on casamino acids, whereas in GSA medium these two components are substituted with MOPS and all

Table II. Components of the different media.

Constituent	GSA (10^{-3} mol·L^{-1})	DN (10^{-3} mol·L^{-1})[d]	Modified DN (10^{-3} mol·L^{-1})
L-alanine	3.4	2.3	3.4
L-arginine	1.1	0.7	1.1
L-asparagine	0.8		0.8
L-aspartate		2.1	1.0
L-cysteine	0.8	0.1	0.8
L-glutamate	2.1	5.9	2.1
L-glutamine	0.7		0.7
Glycine	2.7	1.1	2.7
L-histidine	0.3	0.6	0.3
L-isoleucine	0.8	1.5	0.8
L-leucine	0.8	2.4	0.8
L-lycine-HCl	1.4	2.2	1.4
L-methionine	0.7	0.5	0.7
L-phenylalanine	1.2	0.7	1.2
L-proline	2.6	3.2	2.6
L-serine	2.9	1.3	2.9
L-threonine	1.7	1.2	1.7
L-tryptophan	0.5	< 0.1	0.5
L-tyrosine	0.3	0.1	0.3
L-valine	0.9	2.2	0.9
NH_4^+	9.5	15	15
K^+	1.86	22	22
Na^+	65	91 (106)[c]	91
Ca^{2+}	0.0005	0.09	0.09
Mg^{2+}	0.52	0.2	0.2
Fe^{2+}	0.01		
SO_4^{2-}	0.29	15.1	15.1
$H_2PO_4^-$	1.3	22	22
HPO_4^{2-}		37	37
Cl^-	61.9	21	21
Acetate	15	15[a]	
Lipoic acid		0.01[a]	0.01
Glucose	50	50	50
MOPS	40		
Tricine	4		
Vitamins	+	+	+
Micronutrients	+	+[b]	+[b]

[a] DN is supplemented with either acetate or lipoic acid.
[b] The micronutrients in DN and modified DN are supplied through the water used (ion-exchanged tap-water) and not as in SA through the MOPS solution.
[c] The Na^+ content of DN depends on whether the medium is supplemented with acetate (106) or with lipoic acid (91), as acetate is supplied as Na acetate.
[d] The values of individual amino acids of the DN medium is based on the typical composition of a casein hydrolysate, and hence there is no detectable asparagine or glutamine.

amino acids except aspartic acid which is present in casamino acids (Tab. II). To clarify what caused this inhibition of strain LM0230 and the pyrimidine mutants, they were grown in a modified DN medium, in which the casamino acids were substituted with defined amino acids in concentrations similar to the ones in GSA medium. To fully mimic the casamino acids of DN medium, aspartate was also added to the modified DN medium in a concentration similar to that of asparagine. The growth of LM0230 and the pyrimidine mutants in modified DN medium was shown to be similar to their growth in DN medium (data not shown). This led us to investigate whether removing aspartate from the modified DN medium would have any effect on the growth of LM0230 and the pyrimidine mutants. When grown in a modified DN medium without aspartate, LM0230 and the pyrimidine mutants were shown to grow with a growth rate similar to that obtainable in GSA (0.25 h^{-1}). We then concluded that the effect on growth rate observed in DN medium was due to exogenous aspartate, and all further experiments were done in GSA medium.

3.2. Effect of aspartate on strain LM0230

L. lactis LM0230 and C2 were grown in the synthetic GSA medium supplemented with aspartate at concentrations ranging from 0 to 2000 μg·mL^{-1}. The growth rates were determined, and the values without aspartate were set to 100% (Fig. 2). It is evident that LM0230 is inhibited by aspartate. It appears that the observed effect of aspartate on the growth of LM0230 can be simulated by a sigmoid curve and that the maximum effect is ~ 20% (Fig. 2). Since the apparent sigmoid curve is approaching a definite positive value, it is most likely a result of the action of one or more enzymes. If the effect were of a purely chemical nature, one would expect that the growth rate would approach zero. To determine whether strain LM0230 had acquired mutations that are responsible for this phenotype during the nitrosoguanidine- and the UV-promoted plasmid and phage curing of the mother strain C2, we also cultivated C2 in medium with aspartate. As can be seen from Figure 2, the growth of C2 is not affected by aspartate.

3.3. Aspartate reduces growth rate of *L. lactis* pyrimidine-requiring mutants

In an experiment similar to that described above, *L. lactis* MG1363, MB40 (*pyrB*), MB6 (*pyrDa* and *pyrK*), and MB301 (*pyrF*) were tested for their sensitivity toward aspartate.

The growth rates of strains MG1363, MB6, MB40, and MB301 at different aspartate concentrations are shown in Figure 2. The growth of MB6 and MB301 is clearly affected by aspartate, whereas MB40 and MG1363 are either only affected slightly or not affected at all. The effects on MB6 and MB301 are similar, and the maximum observed reduction in the growth rate, when compared to media without aspartate, is as much as 35–40% at an aspartate concentration of 1000 μg·mL^{-1}. The reduction is 30–35% at 400 μg·mL^{-1} aspartate, and at an aspartate concentration above 1000 μg·mL^{-1}, no further growth inhibition is observed.

Because MB6 (*pyrDa* and *pyrK*) and MB301 (*pyrF*) are pyrimidine auxotrophs, the inhibitory effect of aspartate might be a consequence of competition for uptake between aspartate and uracil. However, this cannot be the case, as the growth rate of MB40 (*pyrB*), which is also a pyrimidine auxotroph, is not affected by the addition of aspartate. We speculate that the observed inhibition is due to the accumulation of the pyrimidine de novo pathway intermediate,

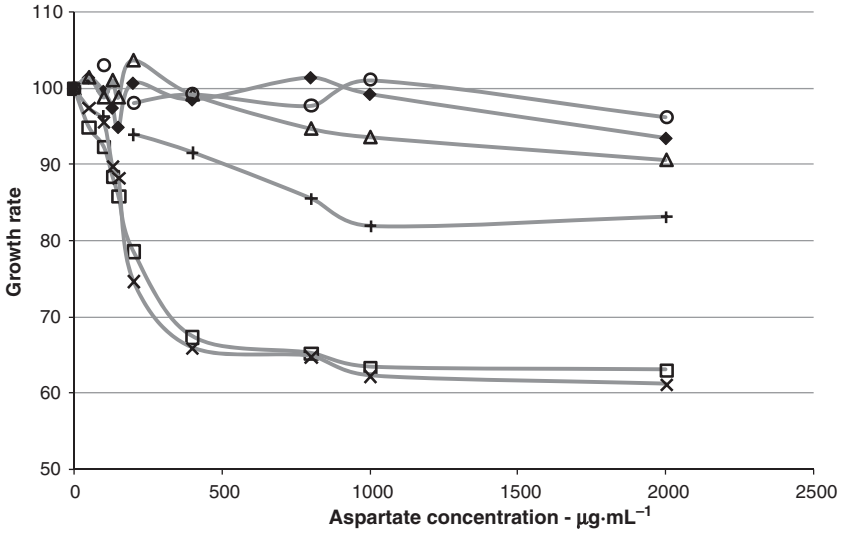

Figure 2. The effect of aspartate on the growth rate of different *L. lactis* subsp. *cremoris* strains in GSA. Growth rates are given as the percentage of the growth rate obtainable in GSA without aspartate. The relative growth rates are the mean values of two independent experiments. The standard deviation was 2.6% or better. ♦, MG1363; □, MB6 (*pyrDa* and *pyrK*); △, MB40 (*pyrB*); ×, MB301 (*pyrF*); +, LM0230; and ○, C2.

carbamoyl aspartate, and that this accumulation is dependent on the combined action of an aspartate transport system and aspartate transcarbamoylase (Fig. 1). As strain MB40 carries a mutation in *pyrB* encoding aspartate transcarbamoylase, this would explain why this strain is not affected by aspartate. The toxicity of carbamoyl aspartate was previously reported in *Salmonella typhimurium* [23], although the mechanism of this toxicity remains unclear. Based on the experiments conducted here, it is not possible to rule out that the accumulation of dihydroorotate is toxic, but the addition of orotic acid to *L. lactis* has no effect on growth [3], and it would be expected that the addition of orotic acid would increase the dihydroorotate pool size, as orotic acid can be converted to dihydroorotate also by the reverse action of the dihydroorotate dehydrogenase activity.

In *E. coli* and *S. typhimurium*, transcription of the pyrimidine de novo genes are subject to complex regulation by UTP and CTP, which results in a decrease in transcription, when pyrimidines are supplied by salvage [18]. Thus, the described toxic effect of carbamoyl aspartate in *S. typhimurium* is only observed during induced expression of the de novo genes: i.e. observed only when the pyrimidine source is the poorly utilized de novo pathway intermediate, orotate [23]. The data presented here indicate that MB6 (*pyrDa* and *pyrK*) and MB301 (*pyrF*) do accumulate pyrimidine de novo intermediates during growth at high aspartate concentrations, despite the fact that uracil is the exogenous pyrimidine source. In turn, this accumulation leads to a consecutive distortion of equilibrium in the reactions between carbamoyl aspartate and dihydroorotate, in the case of MB6, and between carbamoyl aspartate and OMP, in the case of MB301, which results in an intercellular accumulation of carbamoyl aspartate.

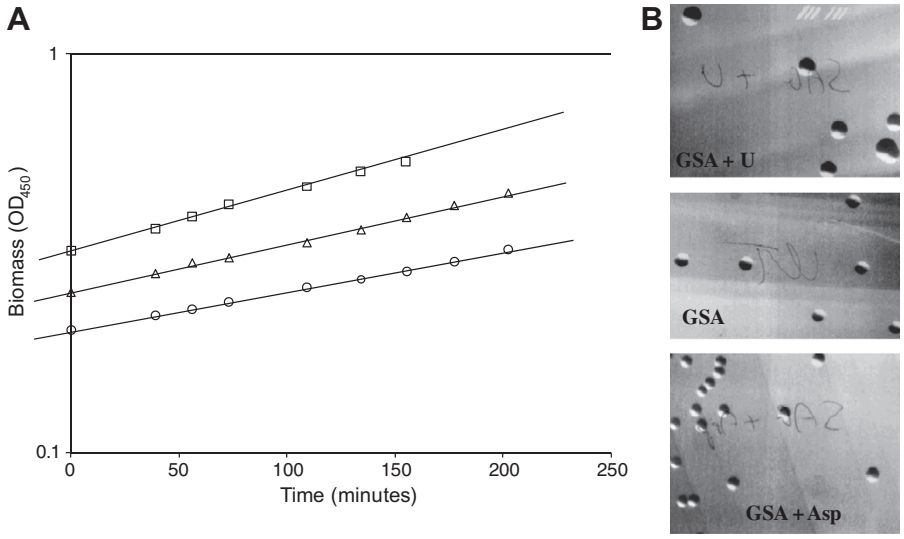

Figure 3. Growth of *L. lactis* LM0230 on GSA, containing either 0.2 mg·mL^{-1} of aspartate (GSA + Asp) or 0.02 mg·mL^{-1} of uracil (GSA + U). (A) A representative example of a growth experiment in liquid medium, supplied with □, uracil; △, nothing; or ○, aspartate. (B) Plates containing same medium. The plates were incubated at 30 °C for 24 h.

3.4. *L. lactis* LM0230 has acquired a partial pyrimidine requirement

In analogy with the observations with the pyrimidine-requiring mutants, we speculated that the observed effect of aspartate on the growth rate of strain LM0230 is due to one or several mutations in genes affecting pyrimidine de novo synthesis, most probably acquired during the process, used to derive this strain from C2. Such mutations could create a bottleneck in de novo pyrimidine synthesis in this strain. To investigate whether strain LM0230 has acquired a partial pyrimidine requirement, we tested the growth of this strain on plates with and without uracil. As is evident from Figure 3, the growth of LM0230 is significantly stimulated by exogenous uracil and (as can also be seen from Fig. 2) inhibited by aspartate. Furthermore, the growth rate of the parental strain of LM0230, C2, is 0.81 ± 0.03 h^{-1} which is equal to that of MG1363 (0.83 ± 0.04 h^{-1}), whereas the growth rate of strain LM0230 when grown in minimal medium (GSA) is only 0.25 ± 0.01 h^{-1}, about 30% of the one observed for the parental strain C2. When grown in media supplemented with pyrimidines, the growth rate of LM0230 is significantly stimulated reaching a growth rate of 0.33 ± 0.01 h^{-1}. These observations indicate that LM0230 has acquired a partial pyrimidine requirement. In addition, it is evident that LM0230 has acquired other partial nutritional requirements, since the addition of uracil can only partially restore wild-type growth.

3.5. Pyrimidine de novo synthesis is affected in strain LM0230

To investigate which mutations in pyrimidine biosynthesis give rise to the observed pyrimidine requirement of strain LM0230, we measured the activity of

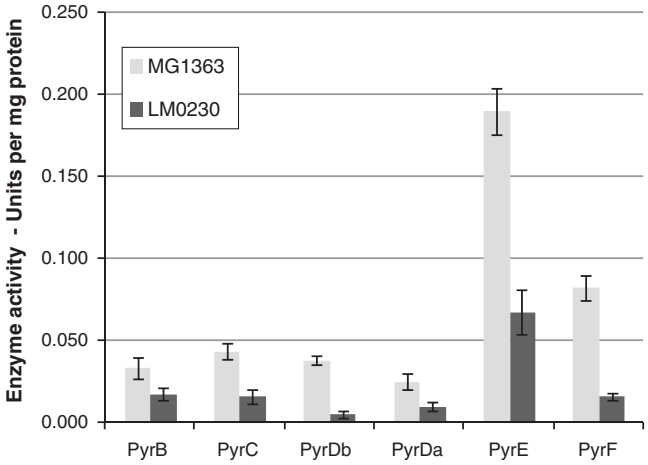

Figure 4. The activities of the enzymes in the pyrimidine biosynthetic pathway. PyrB, aspartate transcarbamoylase; PyrC, dihydroorotase; PyrDa, dihydroorotate dehydrogenase A; PyrDb, dihydroorotate dehydrogenase B complex; PyrE, orotate phosphoribosyltransferase; PyrF, OMP decarboxylase activities were determined in LM0230 (dark gray columns) and MG1363 (light gray columns). The activity of the individual enzymes is given in units per milligram protein in crude extracts. One unit is defined as 10^{-9} mol substrate converted in 1 min.

the pyrimidine de novo enzymes, aspartate transcarbamoylase (PyrB), dihydroorotase (PyrC), dihydroorotate dehydrogenase (PyrDa), dihydroorotate dehydrogenase complex B (PyrDb/PyrK), orotate phosphoribosyltransferase (PyrE), and orotidylate decarboxylase (PyrF). The activity levels were comparable in strains NCDO712 and MG1363 (data not shown), indicating that MG1363 harbors wild-type levels of the pyrimidine de novo synthetic enzymes. The measured activity of the individual enzymes in strains LM0230 is shown in Figure 4. As can be seen, the activities of all the pyrimidine de novo enzymes are significantly lower in LM0230 compared to MG1363. Moreover, the activities of the enzymes of the distal part of the pathway including dihydroorotate dehydrogenase B, orotate phosphoribosyltransferase, and orotidylate decarboxylase are reduced further. This could account for the existence of a bottleneck in the distal part of the pathway, and consequently, when LM0230 is grown in media supplemented with aspartate; this would result in an enhanced incorporation of aspartate, not accompanied by a corresponding increased total flow through the pyrimidine de novo pathway. The observed lowered activity of dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidylate decarboxylase in LM0230 also explains the partial pyrimidine requirement of this strain. However, the data do not clearly indicate which mutations in LM0230 could be responsible for the observed phenotype, since an elucidation of this would require sequencing of all genes encoding pyrimidine de novo enzymes in this strain. The observed partial uracil requirement and the measured enzyme levels can be explained either by mutations in the involved enzymes or by a mutation affecting the expression of all de novo genes eventually by altering the pyrimidine regulator, PyrR, known to be

responsible for regulating all genes in the biosynthetic pathway by an attenuator mechanism [2, 10, 15, 16].

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