Regulation of aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase in *Lactobacillus plantarum*

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The use of a lysine-overproducing strain of *Lactobacillus plantarum* in food or feed fermentations may lead to the production of lysine-rich products. The availability of functional genes and information on the regulation of lysine biosynthesis are required to develop a lysine-overproducing strain. The genome sequence of *L. plantarum* revealed putative lysine biosynthetic genes, some of which may produce isozymes. This study examined the functionality of the genes and the regulation of the first four enzymes of lysine biosynthesis, together with homoserine dehydrogenase, in *L. plantarum*. The genes were expressed in *Escherichia coli*, and the regulation of the enzymes was studied in cell extracts of both recombinant *E. coli* and *L. plantarum*. Among seven lysine biosynthetic genes studied (aspartokinase genes, *thrA1* and *thrA2*; aspartate semialdehyde dehydrogenase genes, *asd1* and *asd2*; dihydrodipicolinate synthase genes, *dapA1* and *dapA2*; and the dihydrodipicolinate reductase gene, *dapB*) plus two homoserine dehydrogenase genes (*hom1* and *hom2*), the products of six genes, i.e. *thrA2*, *asd2*, *dapA1*, *dapB*, *hom1* and *hom2*, showed obvious enzyme activities *in vitro*. The product of one of the homoserine dehydrogenase genes, *hom1*, exhibited both homoserine dehydrogenase and aspartokinase activities. However, the aspartokinase activity was mainly due to ThrA2 and was inhibited by L-lysine and repressed by L-threonine, and the homoserine dehydrogenase activity was mainly due to Hom2 and was inhibited by L-threonine. The aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase were not regulated by the end-products of the pathway.

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INTRODUCTION

L-Lysine is an essential amino acid that has to be available in sufficient amounts in feeds to meet the nutritional requirements of the animals. Plant-based feeds such as crops, forages and silage are usually poor in lysine. Therefore, supplementation with a lysine-rich source is sometimes necessary. The use of lysine-overproducing *Lactobacillus plantarum* in preparing feedstuffs such as silage might improve the utility of the feed.

In order to increase lysine formation in organisms by molecular techniques, information on the regulation of lysine biosynthesis and the availability of the functional genes

is required. L-Lysine, together with other members of the aspartate family of amino acids, L-methionine, L-threonine and L-isoleucine, is synthesized via a branched pathway. The consecutive actions of the first two enzymes, aspartokinase and aspartate semialdehyde dehydrogenase, lead to the formation of aspartate semialdehyde, the precursor of all members of the aspartate family. In the lysine branch, aspartate semialdehyde is converted to dihydrodipicolinate by dihydrodipicolinate synthase, which is then reduced to piperidine dicarboxylate by dihydrodipicolinate reductase (Umbarger, 1978). The first four steps of lysine biosynthesis from aspartate are well conserved in bacteria. From piperidine dicarboxylate, however, there are three variants of the pathway: the succinylase pathway, which uses a succinyl residue as the blocking group, is present in *Escherichia coli* (Kindler & Gilvarg, 1960); the acetylase pathway, involving acetylated intermediates, is found in certain *Bacillus* species (Sundharadas & Gilvarg, 1967; Weinberger & Gilvarg,

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1970); and the dehydrogenase pathway, in which piperidine dicarboxylate is converted in a single step to the ultimate lysine precursor *meso*-diaminopimelate, is found in *Bacillus sphaericus* (Misono *et al.*, 1979; White, 1983).

The regulation of lysine biosynthesis varies among organisms. Only aspartokinase is regulated for the biosynthesis of lysine in *Corynebacterium glutamicum*. It is inhibited by L-lysine plus L-threonine; either L-lysine or L-threonine alone does not inhibit aspartokinase (Nakayama *et al.*, 1966; Cremer *et al.*, 1988). In *E. coli*, many enzymes of lysine biosynthesis are regulated by the end-products of the pathway. There are three isozymes of aspartokinase in *E. coli*: aspartokinase I is repressed by L-threonine plus L-isoleucine and inhibited by L-threonine, aspartokinase II is repressed by L-methionine (Patte *et al.*, 1967), and aspartokinase III is repressed and inhibited by L-lysine (Theze *et al.*, 1974). Aspartokinase I and II are bifunctional enzymes and show both aspartokinase and homoserine dehydrogenase activities. The aspartate semialdehyde dehydrogenase is repressed by L-lysine in *E. coli* (Cohen & Patte, 1963), as is diaminopimelate decarboxylase, the enzyme that catalyses the last step in the pathway (White, 1976). The activity of dihydrodipicolinate synthase in *E. coli* is inhibited by L-lysine (Yugari & Gilvarg, 1962).

In *L. plantarum*, only the regulation of aspartokinase has been reported to date (Adebawo *et al.*, 1997); that study was done with cell extract of *L. plantarum*. Because aspartokinase in *L. plantarum* may be present as an isozyme, as indicated in the genome sequence (Kleerebezem *et al.*, 2003), the study of enzyme regulation using cell extracts of *L. plantarum* may not be appropriate; the aspartokinases should be separated when the regulation of the enzyme is studied. The genome sequence of *L. plantarum* also reveals that aspartate semialdehyde dehydrogenase and dihydrodipicolinate synthase may be present as isozymes.

We studied here the expression of genes encoding the four key enzymes in lysine biosynthesis, i.e. aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase, and the effects of the aspartate family of amino acids on those enzymes in *L. plantarum*. The first step of the pathway and the branching point are potential sites of regulation of carbon flow. Homoserine dehydrogenase was included in this study because this enzyme catalyses the reaction at the branch point and may have a role in the regulation of carbon flow toward L-lysine.

METHODS

Bacterial strains. *L. plantarum* NCIMB 8826 is the parent of strain WCFS1, used in the genome project. It was maintained in MRS medium (Difco). *E. coli* mutants (strains Gif 102, Hfr3000 U482, AT997 and AT999) were maintained in LB medium supplemented with 50 µg *meso*-diaminopimelate ml⁻¹. Strain Gif 102 is deficient in both the aspartokinases and homoserine dehydrogenases. Strains Hfr3000 U482, AT997 and AT999 are deficient in aspartate

semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase, respectively. For preparation of cell extracts, *L. plantarum* was grown in synthetic medium according to Morishita *et al.* (1981), and *E. coli* strains were grown in M9 medium.

Gene cloning and expression of the genes. Lysine biosynthetic genes were amplified from chromosomal DNA of *L. plantarum* NCIMB 8826 by PCR. The primers were designed based on the genome sequence of *L. plantarum* and to include the putative promoter sites of the genes. The primers used were ThrA1-1 (5'-TTCAATA-AGGACTACTTCTTG-3'), ThrA1-2 (5'-ATCTAGGTCAACAAAAA-AGC-3'), ThrA2-1 (5'-AATTAAATTCTCGTGAGTGG-3'), ThrA2-2 (5'-AACACGGCCATAAAAAATG-3'), Hom1-1 (5'-TTAGCAAC-GTCGCTGATG-3'), Hom1-2 (5'-TGATATCTACGTTCTCGTG-3'), Hom2-1 (5'-GGGCCAATTAAATTAGTCTG-3'), Hom2-2 (5'-ACAC-TTGTAGCGTTAGTTGC-3'), Asd1-1 (5'-CATAGGAAGAAAAAG-TGCG-3'), Asd1-2 (5'-AGCCTCCTCAATAATTCTCC-3'), Asd2-1 (5'-ATCTGCGTGTGCAAAACAG-3'), Asd2-2 (5'-GGCAGTCAGCT-TTTCATTAC-3'), DapA1-1 (5'-GGTTAGCCGTGATTGTTG-3'), DapA1-2 (5'-AACTGAAGTCCGGAATCAC-3'), DapA2-1 (5'-ATG-GCATCACACAACTGTC-3'), DapA2-2 (5'-CTGATCCCTGATGA-TGAAAC-3'), DapB-1 (5'-AATAGTGTTTCGACCGAGCG-3') and DapB-2 (5'-AATATCGACGTCATGTAGGGG-3').

The PCR products were cloned into pCR2.1-TOPO (Invitrogen) to facilitate sequencing of the genes. The genes with the right sequences were then subcloned to pUC18 for heterologous expression of the genes in *E. coli* mutants. Transformation of pUC18 harbouring the relevant gene into *E. coli* cells was performed by electroporation using a Bio-Rad Gene Pulser II as described by Dower *et al.* (1988). Aspartokinase and homoserine dehydrogenase genes were expressed in *E. coli* Gif 102, whereas aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase genes were expressed in *E. coli* strains Hfr3000 U482, AT997 and AT999, respectively.

RT-PCR. Nucleotide sequences inside the genes were used as primers for RT-PCR. RNA of *L. plantarum* was used as a template. The RNA was isolated by RNeasy (Qiagen). The primers used were thrA1-RTF (5'-AAAGTTCGGCGGCACGTGAG-3'), thrA1-RTR (5'-GAAACTTTAGCACAGTTCGC-3'), thrA2-RTF (5'-GGCAGCTGGT-AAACGGTTCG-3'), thrA2-RTR (5'-AGTAGTCGTGCGTAAAGTAA-3'), hom1-RTF (5'-TTCAGTTACCGATTGGGACG-3'), hom1-RTR (5'-TGCAACATTTATGGGAGATG-3'), hom2-RTF (5'-AGTTGATT-TGGGGGACGTTTC-3'), hom2-RTR (5'-TTATAACTCGCTAATAGT-TG-3'), asd1-RTF (5'-GTTGATCAGTCGCTGAATC-3'), asd1-RTR (5'-CTATTCAAATAATACCGGTG-3'), asd2-RTF (5'-ATCAAAATG-GTGGAACAGAC-3'), asd2-RTR (5'-TAAGCCCATCTTGTCCATTG-3'), dapA1-RTF (5'-CGAAAAGCGTTAGCAAGTT-3'), dapA1-RTR (5'-CCACCGGCTGACCCAAATGA-3'), dapA2-RTF (5'-TTTCCAC-CGCTTAGAGCAAC-3'), dapA2-RTR (5'-CGACTAACCAGTGTT-GGGCG-3'), dapB-RTF (5'-GTGGTAAAGTAATTGTTGC-3'), and dapB-RTR (5'-TCATAGTAAGTGCTCCAAAC-3').

Preparation of cell extracts and enzyme assay. Cells were harvested by centrifugation, washed twice with deionized water, and resuspended in the buffer used for the enzyme assay. The cells were lysed by sonication (Kaijo Denki Co.) at maximum setting, for 2 min for *E. coli* and 20 min for *L. plantarum*, and the cell debris was separated by centrifugation at 10 000 g for 30 min. The supernatant was used as a cell-free extract.

Aspartokinase was measured as described by Black & Wright (1955b). The assay mixture contained 50 mM Tris buffer pH 7.0, 15 mM ATP, 10 mM MgSO₄, 200 mM (NH₄)₂SO₄, 500 mM hydroxylamine, 25 mM L-aspartate, and cell extract. After incubation at 37 °C for 1 h, the reaction was stopped by addition of FeCl₃. The reaction mixture was centrifuged at 12 000 r.p.m for 5 min and the absorbance

of the supernatant was measured at 540 nm. The absorbance of the samples was compared to those of standards obtained with aspartyl hydroxamate. Activity is expressed as the amount of enzyme required to form 1 nmol hydroxamic acid min^{-1} .

Homoserine dehydrogenase activity was assayed as described by Black (1962) at 30 °C in 1 ml 100 mM potassium phosphate buffer pH 7.0 containing 0.25 mM NADPH and 0.35 mM L-aspartate semialdehyde as substrate. The activity was determined by following the decrease in absorbance at 340 nm. Activity is expressed as the amount of enzyme that oxidized 1 nmol NADPH min^{-1} .

Aspartate semialdehyde dehydrogenase was measured as described by Hegeman *et al.* (1970) in the reverse of the biosynthetic reaction. The assay mixture consisted of 30 mM diethanolamine buffer pH 9.0, 0.8 mM NADP, 40 mM Na_2HAsO_4 , 120 mM NaCl, 0.3 mM L-aspartate semialdehyde, and cell extract. L-Aspartate semialdehyde was synthesized by ozonization of allylglycine as described by Black & Wright (1955a). The rate of reduction of NADP^+ to NADPH was measured spectrophotometrically at 30 °C and 340 nm. Activity is expressed as the amount of enzyme required to form 1 nmol NADPH min^{-1} .

Dihydrodipicolinate synthase was assayed according to the method of Yamakura *et al.* (1974). The assay mixture consisted of 200 mM Tris/HCl buffer pH 8.0, 4 mM sodium pyruvate, 2 mM L-aspartate semialdehyde, and cell extract. After incubation at 37 °C for 10 min, the 1 ml assay mixture was mixed with 1 ml 1 M HCl and 1 ml *o*-aminobenzaldehyde (2 mg ml^{-1} in ethanol). The assay mixture was incubated at 37 °C for an additional 30 min in the dark and then centrifuged. The absorbance of the supernatant was determined at 540 nm. Activity is expressed as the amount of enzyme required to convert 1 nmol aspartate semialdehyde min^{-1} .

Dihydrodipicolinate reductase was assayed by a coupled method as described by Cremer *et al.* (1988). The gene encoding DHPS from *L. plantarum* IAM 12477 was transformed into DHPR-deficient *E. coli* AT999, and the extract of this strain was used to synthesize the reductase substrate, i.e. 2,3-dihydrodipicolinate. The reaction mixture contained 100 mM Tris/HCl buffer pH 7.5, 5 mM sodium pyruvate, 1.5 mM L-aspartate semialdehyde, 1 mM NADPH, and cell extract of *E. coli* AT999 containing 0.4 units of DHPS. After incubation at 30 °C for 10 min, the reductase reaction was started by addition of the cell extract. The NADPH-dependent reduction was followed at 340 nm and 30 °C. Activity is expressed as the amount of enzyme required to oxidize 1 nmol NADPH per minute.

Specific activity of the enzymes is expressed as the activity of the enzyme per mg protein. Protein was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Although the genome sequence of *L. plantarum* is available, there has to our knowledge been no report on the functionality of the genes involved in lysine biosynthesis. In addition, information on the regulation of lysine biosynthesis in this bacterium is limited. The first four enzymes of lysine biosynthesis, i.e. aspartokinase, aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase, were examined in this study; the first three of these enzymes may be present as isozymes, as indicated by the genome sequence. These enzymes include the first enzyme in the pathway and the enzymes at the branch point, which may be involved in the regulation of

carbon flow in lysine biosynthesis. For this reason, homoserine dehydrogenase, which can also catalyse the reaction at the branch point, was included in this study. Therefore, nine genes were cloned from *L. plantarum* DNA and heterologously expressed in an *E. coli* mutant deficient in the corresponding enzyme. Regulation of the enzymes was studied in cell extracts of both *L. plantarum* and the recombinant *E. coli*.

Cloning and expression of lysine biosynthetic genes

Seven genes involved in lysine biosynthesis, i.e. *thrA1*, *thrA2*, *asd1*, *asd2*, *dapA1*, *dapA2* and *dapB*, together with homoserine dehydrogenase genes *hom1* and *hom2*, were amplified by PCR from the DNA of *L. plantarum* NCIMB 8826. The primers used to amplify the genes were designed to include the predicted promoter regions of the genes. The cloned genes with nucleotide sequence identical to the one reported in the genome project were used for further work.

The expression of the genes in *L. plantarum* was determined by RT-PCR. The results showed that six genes (*thrA1*, *thrA2*, *hom2*, *asd2*, *dapA1* and *dapB*) were strongly expressed to mRNA, and the other genes (*hom1*, *asd1* and *dapA2*) were weakly expressed (Fig. 1). The enzyme activities encoded by the cloned genes were assayed in cell extracts of *E. coli* harbouring the gene. As a control, cell extract of *E. coli* harbouring pUC18 was used. For *E. coli* harbouring aspartokinase or homoserine dehydrogenase genes, the cell extract was assayed for both aspartokinase and homoserine dehydrogenase activities. The aspartokinase activity of the cell extract of *E. coli* Gif 102 harbouring *thrA2* was considerably higher than the control (Table 1). However, the aspartokinase activity of the cell extract of *E. coli* Gif 102 harbouring *thrA1* was not significantly different from the control, although *thrA1* was strongly expressed to mRNA in *L. plantarum*. There were two homoserine dehydrogenase genes indicated in the genome sequence of *L. plantarum*; *hom2* was strongly expressed to mRNA, but the expression of *hom1* was weak. However, activity of homoserine dehydrogenase was detected in the cell extract of *E. coli* Gif

Fig. 1. RT-PCR of *thrA1* (lane 1), *thrA2* (lane 2), *hom1* (lane 3), *hom2* (lane 4), *asd1* (lane 5), *asd2* (lane 6), *dapA1* (lane 7), *dapA2* (lane 8) and *dapB* (lane 9) of *L. plantarum*. Lane M, size standards.

Table 1. Enzyme activities in cell extracts of *E. coli* mutants harbouring lysine biosynthesis genes from *L. plantarum*

Activities of aspartokinase (AK), homoserine dehydrogenase (HDH), aspartate semialdehyde dehydrogenase (ASADH), dihydrodipicolinate synthase (DHPS) and dihydrodipicolinate reductase (DHPR) in cell extracts of *E. coli* mutants harbouring the corresponding gene from *L. plantarum*. Results are means $\pm$ SD ($n \geq 3$); ND, not detectable.

Enzyme	Gene	Specific activity (nmol min ⁻¹ mg ⁻¹)	
		<i>rE. coli</i> *	Control†
AK	<i>thrA1</i>	3.6 $\pm$ 0.8	4.0 $\pm$ 0.8
AK	<i>thrA2</i>	162.1 $\pm$ 15.7	4.0 $\pm$ 0.8
AK	<i>hom1</i>	12.8 $\pm$ 2.9	4.0 $\pm$ 0.8
AK	<i>hom2</i>	4.2 $\pm$ 0.5	4.0 $\pm$ 0.8
HDH	<i>thrA1</i>	2.7 $\pm$ 0.7	2.4 $\pm$ 0.8
HDH	<i>thrA2</i>	1.9 $\pm$ 0.3	2.4 $\pm$ 0.8
HDH	<i>hom1</i>	7.6 $\pm$ 1.5	2.4 $\pm$ 0.8
HDH	<i>hom2</i>	1667.0 $\pm$ 380.0	2.4 $\pm$ 0.8
ASADH	<i>asd1</i>	215 $\pm$ 9	207 $\pm$ 15
ASADH	<i>asd2</i>	2149 $\pm$ 173	207 $\pm$ 15
DHPS	<i>dapA1</i>	125.2 $\pm$ 21.2	ND
DHPS	<i>dapA2</i>	ND	ND
DHPR	<i>dapB</i>	1153 $\pm$ 255	23 $\pm$ 12

**E. coli* harbouring the gene from *L. plantarum*.

†*E. coli* harbouring pUC18.

106 harbouring *hom1*, although it was relatively low. The homoserine dehydrogenase activities in the cell extracts of *E. coli* Gif 106 harbouring *hom1* and *hom2* were 7.6 and 1667 nmol min⁻¹ mg⁻¹, respectively, compared to 2.4 nmol min⁻¹ mg⁻¹ for the control. Surprisingly, one of the homoserine dehydrogenase genes, *hom1*, also exhibited aspartokinase activity, although the activity (12.8 nmol min⁻¹ mg⁻¹) was considerably lower than that of ThrA2 (162.1 nmol min⁻¹ mg⁻¹). Thus, Hom1 was a bifunctional enzyme, with both aspartokinase and homoserine dehydrogenase activities. There are two copies of aspartate semialdehyde dehydrogenase genes in *L. plantarum* as shown in the genome sequence. The RT-PCR data showed that only one of them, i.e. *asd2*, was strongly expressed to mRNA. The enzyme activity assay also showed that only Asd2 exhibited strong aspartate semialdehyde dehydrogenase activity. The aspartate semialdehyde dehydrogenase activity of Asd1 was not significantly different from that of the control. The aspartate semialdehyde dehydrogenase activities of cell extract of *E. coli* Hfr3000 U482 harbouring *asd1* and *asd2* were 215 and 2149 nmol min⁻¹ mg⁻¹, respectively, compared to 207 nmol min⁻¹ mg⁻¹ for the control. Although there are two genes encoding dihydrodipicolinate synthase in *L. plantarum* as indicated in the genome sequence, only *dapA1* showed strong expression on RT-PCR and also gave detectable activity (125.2 nmol min⁻¹ mg⁻¹) of dihydrodipicolinate synthase in the cell extract of the recombinant

E. coli. Dihydrodipicolinate synthase activity in the cell extract of *E. coli* AT997 harbouring *dapA2* was undetectable, as it was in the control. The genome sequence of *L. plantarum* shows only one copy of the *dapB* gene. This gene was able to be expressed in *E. coli* AT999, giving dihydrodipicolinate reductase activity of 1153 nmol min⁻¹ mg⁻¹ in the cell extract.

Regulation of enzymes by amino acids

The inhibition study of lysine biosynthetic enzymes was carried out in cell extracts of both *L. plantarum* and recombinant *E. coli*, and the repression study was performed in the cell extract of *L. plantarum*. The repression of some genes was also studied using RT-PCR. In the inhibition study, the amino acid was added to the reaction system, whereas in the repression study, the amino acid was added to the growth medium.

Aspartokinase and homoserine dehydrogenase. Although two enzymes exhibited aspartokinase activity, only the activity of ThrA2 was inhibited by the end-product of the pathway. L-Lysine inhibited the activity of ThrA2 by 87% at 1 mM (data not shown) and by 96% at 10 mM (Table 2). The concerted inhibition of aspartokinase by L-lysine plus L-threonine as found in *C. glutamicum* was not observed in *L. plantarum* (data not shown). Study of the regulation of aspartokinase using *L. plantarum* cell extract showed inhibition of the enzyme by L-lysine and repression by L-threonine (Table 3). The repression of aspartokinase by L-threonine in *L. plantarum* may contribute to the reduced growth rate when the cells were grown in the medium supplemented with L-threonine (Fig. 2).

The inhibition and repression effects observed in the cell extract of *L. plantarum* are probably attributable to ThrA2, based on the fact that the aspartokinase activity of Hom1 was considerably lower than that of ThrA2. Our results are thus at variance with the report of Adebawo *et al.* (1997), who found that aspartokinase in *L. plantarum* was not inhibited by amino acids of the aspartate family. Despite that difference, the activation of aspartokinase by L-methionine observed in this study (Tables 2 and 3) was also reported by Adebawo *et al.* (1997). The regulation of aspartokinase in *L. plantarum* was found to differ from that in other organisms such as *E. coli* (Patte *et al.*, 1967; Theze *et al.*, 1974), *Bacillus subtilis* (Graves & Switzer, 1990; Rosner & Paulus, 1971) and *C. glutamicum* (Nakayama *et al.*, 1966).

The activity of homoserine dehydrogenase in *L. plantarum* was contributed by Hom1 and Hom2, although the activity of Hom1 was considerably lower than that of Hom2. The activity of Hom1 was not inhibited by amino acids of the aspartate family, though the activity of Hom2 was inhibited by L-threonine; at 1 mM L-threonine, the homoserine dehydrogenase activity of Hom2 was reduced by 90% (data not shown), and at 10 mM L-threonine, the activity of Hom2 was inhibited completely (Table 2). The inhibition of homoserine dehydrogenase by L-threonine was also

Table 2. Inhibition of aspartokinases and homoserine dehydrogenases by amino acids of the aspartate family

Studies were carried out in the cell extracts of *E. coli* harbouring the relevant gene from *L. plantarum*. L-Amino acid was added to the assay system to reach a final concentration of 10 mM. Results are means $\pm$ SD ($n \geq 3$).

Amino acid	Aspartokinase (nmol min ⁻¹ mg ⁻¹)		Homoserine dehydrogenase (nmol min ⁻¹ mg ⁻¹)	
	ThrA2	Hom1	Hom1	Hom2
–	162.1 $\pm$ 15.7	12.8 $\pm$ 2.9	7.6 $\pm$ 1.5	1667.0 $\pm$ 380.0
Lysine	9.6 $\pm$ 1.9*	12.7 $\pm$ 3.1	6.5 $\pm$ 0.6	1754.0 $\pm$ 450.0
Methionine	290.8 $\pm$ 71.2*	12.2 $\pm$ 2.3	8.1 $\pm$ 1.6	1817.0 $\pm$ 510.0
Threonine	196.9 $\pm$ 38.0	12.3 $\pm$ 4.9	6.8 $\pm$ 0.9	9.9 $\pm$ 9.6*
Isoleucine	289.0 $\pm$ 64.3*	13.0 $\pm$ 2.9	8.6 $\pm$ 1.2	1655.0 $\pm$ 270.0

*Student's *t*-test showed that the enzyme activities were significantly different from the control (activity with no inhibition) at 95 % confidence interval.

observed when we used cell extract of *L. plantarum* (Table 3).

Aspartate semialdehyde dehydrogenase. Study of the regulation of aspartate semialdehyde dehydrogenase in the cell extracts of both *E. coli* Hfr3000 U482 harbouring *asd2* and *L. plantarum* did not show any influence by amino acids of the aspartate family. Such a lack of effect was also observed in *C. glutamicum* (Cremer *et al.*, 1988). However, aspartate semialdehyde dehydrogenase in *E. coli* is repressed by L-lysine (Cohen & Patte, 1963).

Dihydrodipicolinate synthase. There was no inhibition of dihydrodipicolinate synthase by any amino acids of the

aspartate family as shown in the study using cell extracts of *E. coli* AT997 harbouring *dapA1*. In addition, the transcription of *dapA1* to mRNA in *L. plantarum* was not repressed when the cells were grown in medium supplemented with 10 mM L-lysine (data of RT-PCR not shown). The absence of inhibition and repression of dihydrodipicolinate synthase by the end-product of the pathway was also found in *Brevibacterium lactofermentum* (Tosaka & Takinami, 1978), *C. glutamicum* (Cremer *et al.*, 1988) and *Bacillus stearothermophilus* (Selli *et al.*, 1994), but different results were found in *E. coli* (Yugari & Gilvarg, 1962) or *Bacillus sphaericus* (Barlett & White, 1986), where dihydrodipicolinate synthase was reported to be inhibited by lysine.

Table 3. Inhibition and repression of aspartokinases and homoserine dehydrogenases by 10 mM of amino acids of the aspartate family

Studies were carried out in cell extracts of *L. plantarum*. In the inhibition study, L-amino acid was added to the assay system to reach a final concentration of 10 mM, whereas in the repression study, L-amino acid was added to the growth medium at a concentration of 10 mM. Results are means $\pm$ SD ($n \geq 3$).

Amino acid	Aspartokinase (μ mol min ⁻¹ mg ⁻¹)	Homoserine dehydrogenase (μ mol min ⁻¹ mg ⁻¹)
Inhibition		
–	8.4 $\pm$ 1.0	2.5 $\pm$ 0.1
Lysine	4.9 $\pm$ 0.5*	2.4 $\pm$ 0.1
Methionine	13.1 $\pm$ 1.5*	2.3 $\pm$ 0.2
Threonine	8.3 $\pm$ 1.7	1.6 $\pm$ 0.4*
Isoleucine	11.4 $\pm$ 2.3	2.2 $\pm$ 0.1
Repression		
–	7.5 $\pm$ 0.7	2.1 $\pm$ 0.6
Lysine	7.4 $\pm$ 0.7	3.8 $\pm$ 0.7
Methionine	5.9 $\pm$ 0.7	1.8 $\pm$ 0.2
Threonine	2.8 $\pm$ 0.9*	1.7 $\pm$ 0.3
Isoleucine	6.3 $\pm$ 0.5	2.5 $\pm$ 0.2

*Student's *t*-test showed that the enzyme activities were significantly different from the control (activity with no inhibition or repression) at 95 % confidence interval.

Fig. 2. Effect of L-threonine on the growth of *L. plantarum*: OD₆₀₀ of cultures growing in minimal medium (▲), minimal medium plus 20 mM L-lysine (○), minimal medium plus 50 mM L-lysine (●), minimal medium plus 20 mM L-threonine (□) and minimal medium plus 50 mM L-threonine (■).

Dihydrodipicolinate reductase. Dihydrodipicolinate reductase in *L. plantarum* was not regulated by amino acids of the aspartate family when it was studied in the cell extracts of both *E. coli* AT999 harbouring *dapB*

and *L. plantarum*. The dihydrodipicolinate reductases in *Br. lactofermentum* and *C. glutamicum* were also not regulated by the end-product of the pathway. However, dihydrodipicolinate reductase in *Staphylococcus aureus* is repressed by lysine (Barnes *et al.*, 1969).

DISCUSSION

The genome sequence of *L. plantarum* indicates the presence of a second copy of genes encoding aspartokinase, aspartate semialdehyde dehydrogenase and dihydrodipicolinate synthase, involved in the synthesis of lysine. However, only one copy of these genes, i.e. *thrA2*, *asd2* and *dapA1*, showed obvious enzyme activity when expressed in *E. coli*. The RT-PCR data showed that those three genes were strongly expressed to mRNA (Fig. 1), whereas the *asd1* and *dapA2* genes, which did not show enzyme activity *in vitro*, were weakly expressed as shown by RT-PCR. The *asd1* and *dapA2* genes might have lost their functions during their evolution. ThrA1 did not show any aspartokinase activity *in vitro*, though the RT-PCR data showed strong expression of *thrA1*. When *thrA1* was cloned to the expression vector pET25b and expressed in *E. coli*, it did not give an enzyme activity (data not shown). It is assumed that the degree of evolution of *thrA1* is different from that of *asd1* or *dapA2*.

Our study of the inhibition of lysine biosynthetic enzymes of *L. plantarum* was carried out in cell extracts of *E. coli*

Fig. 3. Lysine biosynthetic pathway and its regulation in *L. plantarum* as determined in this study.

mutants harbouring the relevant gene and of *L. plantarum*. However, we studied repression of enzyme synthesis in cell extract of *L. plantarum* only. Repression of foreign enzymes in *E. coli* may not represent the repression of enzyme synthesis in the species from which the enzymes originate. However, a study of repression of an enzyme in its own cell extract is suitable only if the enzyme is not an isozyme. In lysine biosynthesis of *L. plantarum*, aspartokinase was regulated by the end-product of the pathway, whereas aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase were not subject to such regulation (Fig. 3). The study using cell extract of *E. coli* Gif 102 harbouring *thrA2* revealed that this aspartokinase was inhibited by L-lysine (Table 2), and the study using the cell extract of *L. plantarum* showed that aspartokinase was inhibited by L-lysine and repressed by L-threonine (Table 3). The aspartokinase repressed by L-threonine is likely to be ThrA2, based on the finding that the activity of Hom1 is considerably lower than that of ThrA2.

The inhibition by L-lysine and repression by L-threonine of ThrA2 is sufficient to control the synthesis of all amino acids of the aspartate family provided that the remaining aspartokinase activity supplies sufficient precursor for the synthesis of the rest of the amino acids. At the branching point, dihydrodipicolinate synthase competes with homoserine dehydrogenase for their common substrate L-aspartate semialdehyde (Fig. 3). However, the flow of L-aspartate semialdehyde at this branching point is controlled by regulating the homoserine dehydrogenase, given that regulation of dihydrodipicolinate synthase has not been observed in *L. plantarum*. The inhibition of Hom2 by L-threonine leads to less conversion of aspartate semialdehyde toward L-threonine, though the formation of aspartate semialdehyde also becomes reduced due to the repression of ThrA2 by L-threonine, thus allowing a balanced flow of intermediate at the branch point.

In *C. glutamicum*, the flow of aspartate semialdehyde to L-lysine is enhanced by increasing the dihydrodipicolinate synthase activity (Eggeling *et al.*, 1998) or by decreasing the homoserine dehydrogenase activity (Vrljic *et al.*, 1995), leading to improved L-lysine production. However, this method of redirecting aspartate semialdehyde flow toward L-lysine may not be applicable to *L. plantarum* because the supply of aspartate may be reduced, due to inhibition of ThrA2 by L-lysine when L-lysine is in excess, unless a mutant strain with L-lysine-insensitive ThrA2 is used.

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