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ORIGINAL PAPER

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Cloning, nucleotide sequencing, and expression of the 3-methylaspartate ammonia-lyase gene from *Citrobacter amalonaticus* strain YG-1002

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Abstract The gene coding for 3-methylaspartate ammonia-lyase (3-methylaspartase, MAL, EC 4.3.1.2) from *Citrobacter amalonaticus* strain YG-1002 (TPU 6323) was cloned onto plasmid pBluescript II KS(+), and the nucleotide sequence of the 1239-bp open reading frame (ORF), consisting of 413 codons, was identified as the *mal* gene coding for MAL. The predicted polypeptide has 62.5% identity with MAL from the obligate anaerobe, *Clostridium tetanomorphum* NCIMB 11547. ORF1, which showed 58.6% and 58.8% identities with subunit E of the glutamate mutases of *C. tetanomorphum* and *Clostridium cochlearium* respectively, was found in the upstream region of the *mal* gene. An expression plasmid pMALCA3 (5.4 kb), in which the *mal* gene was expressed under control of the *lac* promoter on the vector, was constructed. With feeding of 1 mM isopropyl β-D-thiogalactopyranoside, the amount of the enzyme in a cell-free extract of the transformant, *E. coli* JM109/pMALCA3, was elevated to 51 800 units/l culture, which is about 50-fold that of *C. amalonaticus* strain YG-1002. It was calculated that the enzyme comprised over 40% of the total extractable cellular proteins. The enzyme produced by the *E. coli* transformant was purified in a crystalline form and shown to be identical to that of the wild-type strain with respect to specific activity, molecular mass, subunit structure, enzymological properties, and N-terminal amino acid sequences.

Introduction

The enzyme 3-methylaspartate ammonia-lyase (3-methylaspartase, MAL, EC 4.3.1.2) is associated with the anaerobic degradation of (*S*)-glutamate in obligate anaerobes and is known to catalyze reversible amination/deamination reactions between mesaconate and (2*S*,3*S*)-3-methylaspartate (Hanson and Havir 1972) (Fig. 1). We have reported on the occurrence (Asano and Kato 1994a; Kato and Asano 1997), purification, and characterization (Asano and Kato 1994b; Kato and Asano 1995a, b) of the enzymes from facultative anaerobic bacteria belonging to the family of Enterobacteriaceae, and applied this information to the synthesis of several 3-substituted (*S*)-aspartates (Asano and Kato 1994a). To employ the enzyme in organic synthesis, we needed to overproduce it by gene technology, because the enzyme is inducibly formed only when the strains are grown statically or anaerobically in the presence of (*S*)-glutamate, when the cell yield is very low.

Recently the high-resolution structure of aspartase, which catalyzes a similar reaction with MAL, was reported by Shi et al. (1997). It is of interest to compare the primary structures of MAL with those of aspartases, but little is known about the amino acid sequence of MAL except for the enzyme in the obligate anaerobic bacteria *Clostridium tetanomorphum* H1 (Goda et al. 1992).

In this paper, we report the cloning, sequencing and expression in *Escherichia coli* of the *mal* gene from *Citrobacter amalonaticus* strain YG-1002, not only to facilitate overproduction of the enzyme for the stereospecific synthesis of a variety of natural and unnatural amino acids, but also to elucidate its structural relationship to other proteins.

Materials and methods

Bacterial strains and plasmids

C. amalonaticus strain YG-1002 (TPU 6323, Asano and Kato 1994; Kato and Asano 1995a) was used as the source of the enzyme and genomic DNA. *E. coli* JM109 and pUC 119 were purchased from Takara Shuzo (Japan) and pBluescript II KS(+) was from Toyobo (Japan). *E. coli* strains were cultivated at 37 °C in Luria Bertani

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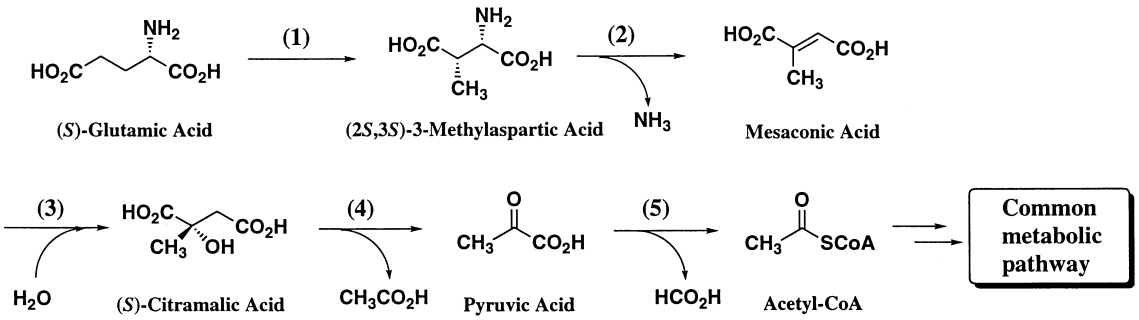

Fig. 1 Anaerobic degradation of (*S*)-glutamate by the mesaconate route. Enzymes shown are (*1*) glutamate mutase, (*2*) 3-methylaspartate ammonia-lyase, (*3*) (*S*)-citramalate hydrolyase, (*4*) citramalate lyase, and (*5*) pyruvate-formate lyase

(LB) medium (Maniatis et al. 1982) with 50 μg/ml ampicillin. Plasmids were purified by a plasmid purification kit (Quiagen) or by automatic plasmid isolation system PI-100 (Kurabo, Osaka, Japan).

Enzyme assay and protein measurement

MAL activity was measured and quantified as described previously (Kato and Asano 1995a, b). Protein was assayed by the method of Bradford (1976) using a dye reagent concentrate (Bio-Rad Laboratories, USA) with bovine serum albumin as the standard, or by measuring the absorbance at 280 nm.

Analytical methods

Polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate (SDS) was carried out as described by Davis (1964) and Laemmli (1970) respectively, with an electrophoresis unit from ATTO (Tokyo, Japan). The molecular mass of the enzyme was estimated as described (Kato and Asano 1995a, b). BLAST and FASTA searches were performed to detect similarities with the amino acid sequence of the enzyme and other known proteins via the NCBI BLAST and FASTA server respectively.

Purification of MAL from *C. amalonaticus* strain YG-1002 and preparation of internal peptides

C. amalonaticus strain YG-1002 was grown statically with medium containing (*S*)-glutamate and the enzyme was purified by ammonium sulfate fractionation and ion-exchange, hydrophobic, and gel-filtration column chromatographies as described (Kato and Asano 1995a). Purified MAL (1 mg) was digested with 15 μg lysyl endopeptidase (Wako, Japan) in 1 ml 15 mM TRIS/HCl (pH 9.0) containing 4 M urea, at 30 °C overnight. The reaction mixture was subsequently eluted by HPLC on an ODS-80Ts column (4.5 × 150 mm) in a 20%–70% CH_3CN linear gradient containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min with continuous monitoring of the absorbance at 215 nm, and each peptide fragment was collected. To determine amino acid sequences, the peptide sample was covalently bound to the Sequelon AA (arylamine) and DITC (diisothiocyanate) membranes as described previously (Kato and Asano 1995a, b), and applied to an automatic protein sequencer (Prosequencer, 6625, Millipore Corp., USA).

Cloning and sequencing of *mal* gene

Oligonucleotide primers were synthesized on the basis of the amino acid sequences of the NH_2 terminus (Kato and Asano 1995a) and internal fragments generated with lysyl endopeptidase. The amino acid sequence MKIKQA (N-terminal peptide) was used to model the oligonucleotide pool 5′-ATGAA(A/G)AT(A/C/T)AA(A/G)CA(A/G)GC-3′ (sense standard) and CHMVQI (internal peptide) to model 5′-AT(C/T)TG(A/C/G/T)ACCAT(A/G)TG(A/G)CA-3′ (antisense standard). The genomic DNA of *C. amalonaticus* strain YG-1002 was isolated by the method of Saito and Miura (1969). DNA was amplified by the polymerase chain reaction (PCR) using a thermal cycler (Perkin-Elmer Cetus Instruments, USA). Reaction mixtures contained 1 μg DNA, 100 pmol each oligonucleotide pool, and *Thermus aquaticus* DNA polymerase (Ex Taq; Takara) in a volume of 50 μl. Thirty-five thermal cycles were employed, each consisting of 95 °C for 1 min, 50 °C for 1.5 min, and 72 °C for 2.5 min. The gel-purified PCR product (600 base pairs, bp) was cloned into the *Sma*I site of pUC 118 replicative-form DNA and designated as pMALCA1. The gel-purified PCR product was further used as a radiolabeled probe to clone the full-length *mal* gene. Hybridizations were carried out as described (Dairi and Asano 1995). A specific positive signal was detected in an *Eco*RV-digested DNA fragment (4.2 kb) with genomic Southern hybridizations. After digestion of genomic DNA with *Eco*RV, the 3.5- to 5.5-kb DNA fragments were purified by agarose-gel electrophoresis and ligated with *Sma*I-digested and dephosphorylated pBluescript II KS(+) by using T4-ligase. The ligated DNA was used to transform *Escherichia coli* JM109 to construct a sub-genomic library of *C. amalonaticus* strain YG-1002. The sub-genomic library was screened by colony hybridization. One positive clone, pMALCA2, carrying the 4.0-kb *Eco*RV fragment, was selected for further analysis. To generate shorter clones suitable for sequencing, the exonuclease III deletion method for the kilo sequencing kit (Takara) was used. The sequencing was done by the dideoxy-DNA chain-termination procedure of Sanger et al. (1977) using an automatic DNA sequencer (ALF, Pharmacia, Sweden).

Expression of MAL in transformants and purification of the enzyme

All enzyme purification was done at 4 °C. Potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol was used throughout the purification process. The 2.3-kb *Nco*I fragment was inserted into *Sma*I sites of the pUC119 to construct pMALCA3, in which the *mal* gene was expressed under the *lac* promotor. A 900-μl sample of the overnight culture of *E. coli* JM109 cells harboring pMALCA3 was transferred into 300 ml LB medium containing 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) in a 2-l conical flask and grown at 37 °C for 20 h.

The washed cells (13 g, wet weight) from 3 l of culture were suspended in 80 ml 0.1 M buffer. The cells were disrupted for 30 min by a Kubota-Shoji 9 kHz ultrasonic oscillator. The disrupted cells were removed by centrifugation at 32000 *g* for 25 min. The supernatant solution was brought to 30% saturation with solid ammonium sulfate. The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant to 60% saturation. The active precipitate collected by centrifugation was dissolved in 0.01 M buffer and dialyzed against the same buffer (10 l × 3). To the enzyme solution was added ammonium sulfate to 25% saturation. The enzyme solution was placed on a column of Butyl-Toyopearl 650 M (3 × 15 cm), equilibrated with 0.01 M

buffer containing ammonium sulfate to 25% saturation. After the column had been washed with 0.01 M buffer containing ammonium sulfate to 25% saturation, the active fractions were eluted with a linear gradient of ammonium sulfate (25%–15% saturation). The active fractions collected were dialyzed and concentrated by ultrafiltration to about 20 mg/ml. Solid ammonium sulfate was carefully added to the enzyme solution until it became slightly turbid, and the mixture then left at 4 °C. Crystallization began after 1 day and was virtually complete within 2 days. The enzyme was isolated in needle form.

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL, GSDB, DDBJ, and NCBI Data Banks with accession number AB005294.

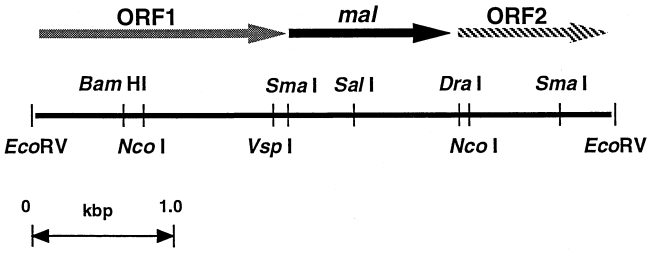

Fig. 2 Restriction map of the insert of pMALCA2

Results

Construction of a gene probe for the *mal* gene of *C. amalonaticus* strain YG-1002

Purified MAL of *C. amalonaticus* strain YG-1002 was digested with lysyl-endopeptidase and several peptides were obtained by HPLC separation. On the basis of the amino acid sequences of N-terminal and internal peptides of MAL, which were determined by Edman degradation, two degenerate oligonucleotides were prepared. They were used to amplify a DNA fragment by PCR with genomic DNA of *C. amalonaticus* as a template. A 0.6-kb PCR product was identified as a part of the *mal* gene by sequencing, since the N-terminal amino acid sequence of MAL was found in the deduced sequence of the amplified DNA.

Cloning and sequencing of the *mal* gene

By genomic Southern hybridization using the PCR product as a probe, specific positive signals were detected in DNA digested with *Ban*III (1.6 kb), *Bss*HII (2.0 kb) and *Eco*RV (4.2 kb). We screened a genomic library constructed by the enzyme digests of genomic DNA and pBluescript II KS(+) by colony hybridization. One positive clone, pMALCA2, carrying a 4.0-kb insert digested with *Eco*RV, was selected for further analysis. Figure 2 shows a restriction map of the insert of pMALCA2. The 1.3-kb *Vsp*I-*Nco*I fragment, which was thought to contain the entire *mal* gene, was prepared from pMALCA2 and used for sequence analysis. The nucleotide sequence of the fragment revealed that there is a single open reading frame (ORF), starting at position 1 and ending at position 1239, and translation of nucleotides, underlined in Fig. 3, gives an amino acid sequence identical to those obtained from the N-terminal and internal peptides. A putative ribosome-binding site (AGGAA) is located 12 bp upstream of the gene. No clear promoter consensus sequence of *Citrobacter* (Mikulskis and Cornelis 1994) is detectable upstream of the *mal* gene. The molecular mass of the encoded MAL protein (45 468 Da), estimated from the deduced amino acid sequence, agreed with the value of 42 kDa determined by SDS-PAGE of the purified wild-type MAL

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MalCA   N-   1   MKIKQALFTAGYSSFYFDDQQAIKNGAGHDGFIYTGDPVTPGFTSVRQAGECVSVQLILENGAVAVGDCA
                 ***   * * * . ****** ***.******* ***  ** *** *** **..** *.**.* ** ****
MalCT   N-   1   MKIVDVLCTPGLTGFYFDDQRAIKKGAGHDGFTYTGSTVTEGFTQVRQKGESISVLLVLEDGQVAHGDCA

MalCA       71   AVQYSGAGGRDPLFLAEHFIPFLNDHI-K---PLLEGRDVDAFLPNARFFDKLRIDGNLLHTAVRYGLSQ
                 ***************  ***     * *    * * **..  * * *  ***. ..** ****.***..*
MalCT       71   AVQYSGAGGRDPLFLAKDFIPV----IEKEIAPKLIGREITNFKPMAEEFDKMTVNGNRLHTAIRYGITQ

MalCA      137   ALLDATALASGRLK-T--EVVCDEWQLPCVPE--AIPLFGQSGDDRYIAVDKMILKGVDVLPHALINNVE
                 *.*** *    * * *  **  **   *   *  *.*.*.*******  *****.*  ************
MalCT      137   AILDAVAKT--R-KVTMAEVIRDEYN-PGA-EINAVPVFAQSGDDRYDNVDKMIIKEADVLPHALINNVE

MalCA      202   EKLGFKGEKLREYVRWLSDRILSLRSSPR--YHPTLHIDVYGTIGLIFDMDPVRCAEYIASLEKEAQGLP
                 **** ***** ***.** ***. **   *  * *  *********  **.*    *.** .*  **   *
MalCT      202   EKLGLKGEKLLEYVKWLRDRIIKLRV--REDYAPIFHIDVYGTIGAAFDVDIKAMADYIQTLA-EAAK-P

MalCA      270   --LYIEGP--V-DAGNKPDQIRMLTAITKELTR--L-GSGVK--IVADEWCNTYQDIVDF-TD--AGSCH
                   * ****  * *   *  *  *  *.   * *  * * **   .********  * * * **  **  *
MalCT      268   FHLRIEGPMDVEDRQ-K--Q--ME-AMRD-L-RAELDGRGVDAELVADEWCNTVED-VKFFTDNKAG--H

MalCA      327   MVQIKTPDLGGIHNIVDAVLYCNKH-GMEAYQGGTCNETEISAR-TC-VHVALAARPMRMLIKPGMGFDE
                 **********. ** ** .** *  ** ** *******  **  *  . .*  ** .  * ***** **
MalCT      327   MVQIKTPDLGGVNNIADAIMYC-KANGMGAYCGGTCNETNRSAEVTTNIGMACGARQV--LAKPGMGVDE

MalCA      394   GLNIVFNEMNRTIALLQT-KD 413
                 *. ** ***** .**.   *
MalCT      394   GMMIVKNEMNRVLALVGRRK  413
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Fig. 3 Comparison of the amino acid sequences of the *mal* gene product from *Citrobacter amalonaticus* strain YG-1002 and 3-methylaspartate ammonia-lyase of *Clostridium tetanomorphum* NCIMB 11547. The first amino acid sequence (*MalCA*) is the sequence of the *mal* product from *C. amalonaticus* strain YG-1002 (sequence size: 413) (this study). Underlined are those amino acid residues that are found in the N-terminal and internal peptide sequences determined with purified MAL of the wild-type strain. The second amino acid sequence (*MalCT*) is that of 3-methylaspartate ammonia-lyase of *C. tetanomorphum* NCIMB 11547 (sequence size: 413). ⋆ Identical residue, • similarity of functional group

(Kato and Asano 1995a). Further sequencing of the flanking regions of the *mal* gene in pMALCA2 revealed two other ORF: ORF1 (1443 bp), which ends 2 bp upstream of the *mal* start codon, and ORF2 (885 bp), which starts 6 bp downstream from it.

Deduced primary sequence and similarities with other proteins

Computer alignments by the SWISS-PROT and NBRF-PIR data bases using the BLAST, FASTA, and DNASIS programs showed that the deduced primary structure of MAL had 70.7% similarity (62.5% identity) with MAL of *C. tetanomorphum* (Goda et al. 1992), as shown in Fig. 3. No homology was seen with the other proteins including other amino acid ammonia-lyases, such as aspartase (Takagi et al. 1986), histidine ammonia-lyase (Wu et al. 1992), and phenylalanine ammonia-lyase (Frank et al. 1991). Since the total number of amino acid residues of *Citrobacter* MAL is identical with that of *Clostridium* (413 amino acids), we compared the *mal* genes of *Citrobacter* and *Clostridium* and found them to be 55% identical at the gene level (data not shown). This result suggests that both MAL enzymes have evolved from the same ancestral gene.

As shown in Fig. 4, the deduced amino acid sequence (481 amino acids) of ORF1 showed 82.0% and 82.6% similarities (58.6% and 58.8% identities) with the subunit E of glutamate mutases of *C. tetanomorphum* (Brecht et al. 1993) and *C. cochlearium* (Zelder et al. 1994) respectively. The amino acid sequences (295 amino acids) encoded by ORF2 did not show any significant similarity to other proteins (data not shown).

Expression of the *mal* gene in *E. coli*

For an expression of the *mal* gene in *E. coli*, pMALCA3, which was constructed by inserting the 2.3-kb *Nco*I fragment into pUC119 in which the *mal* gene was expressed under the control of the *lac* promotor, was used. Strong enzyme activity was detected in the cells of the recombinant *E. coli* harboring the plasmid, when grown aerobically in LB medium containing 1 mM IPTG at 37 °C. After 20 h, the production of MAL reached a maximum, with an activity of over 50 000 units/l culture (100 units/mg protein). The transformants did not show any activities in the absence of IPTG. The same enzyme activity as for the cell-free extract was detected in the

Fig. 4 Comparison of the amino acid sequences of the ORF1 product from *Citrobacter amalonaticus* strain YG-1002 and subunit E of glutamate mutases of *Clostridium tetanomorphum* NCIMB 11547 and *Clostridium cochlearium* DSM 1285. The first (*GlmL*) and third (*MutL*) amino acid sequences are the subunit E of glutamate mutases of *C. tetanomorphum* NCIMB 11547 (sequence size: 485) and *C. cochlearium* DSM 1285 (sequence size: 487) respectively. The second amino acid sequence (*GlmCA*) is the sequence of the ORF1 product from *C. amalonaticus* strain YG-1002 (sequence size: 481) (this study). ⋆ Identical residue, • similarity of functional group

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GlmL    N- 1   MELKNKKWTDEEFHKQREEVLQQWPTGKEVD-LQEAVDYLKKIPAEKNFAEKLVLAKKKGITMAQPRAGVALLDEHIELLRYLQDEGGADFLPSTIDAYTRQ
               ***.*** * .**  .*..*** * ***.*. ....* * . ** .* *.. *. * ..* *..********.*******. ** *   *.**********
GlmCA   N- 1   MELRNKKLTHDEFMTERHQVLQTWHTGKDVEHFEDGVKYQQTIPEKKRFSHALLKADQEGKTLSQPRAGVALMDEHIELLKTLQVE--CDLLPSTIDAYTRL
               ***.*** * .**. .*..**. * ***.*. ....* * . .* .* *.  *..* . * **.********.****.**. ** *   ************
MutL    N- 1   MELKNKKWTDEEFFKQREEVLKQWPTGKEVD-LQEAVDYLKKVPTEKNFADKLVRAKEAGITLAQPRAGVALLDEHINLLRYLQDEGGADLLPSTIDAYTRQ

GlmL     102   NRYDECENGIKESEKAGRSLLNGFPGVNYGVKGCRKVLEAVNLPLQARHGTPDSRLLAEIIHAGGWTSNEGGGISYNVPYAKNVTIEKSLLDWQYCDRLVGF
               ***.*   **..* .** * ***.* **.** .**.. **.. *.*.******.******  *.*.** ********.**** **.***. *********.*.
GlmCA    101   NRYEEAAIGIQKSIEAGTSKLNGLPVVNHGVAACRRMTEALEKPIQVRHGTPDARLLAEIAMASGFTSYEGGGISYNIPYAKRVTLEKSIRDWQYCDRLMGL
               *****  ***..* .** * ***.* ***** .**.. *... *.*.******.******  *.*.** ************* * ..* ..*********.*.
MutL     102   NRYEECEIGIKESEKAGRSLLNGFPGVNHGVKGCRKVLESVNLPLQARHGTPDSRLLAEIIHAGGWTSNEGGGISYNIPYAKSVPIDKCLKDWQYCDRLVGF

GlmL     204   YEEQGVHINREPFGPLTGTLVPPSMSNAVGITEALLAAEQGVKNITVGYGECGNMIQDIAALRCLEEQTNEYLKAYGYNDVFVTTVFHQWMGGFPQDESKAF
               ***.*..**************.** **.**.* *.*** *****.******. *.. *****.. *.* ..**.  **..*  ..************.******
GlmCA    203   YEEHGIRINREPFGPLTGTLIPPFMSHAVAIIEGLLALEQGVKSITVGYGQVGSLTQDIAAIQSLRELSHEYFQNYGFSDYELSTVFHQWMGGFPEDESKAF
               ***.*..**************.** **.**.* *.*** *****.******. *.. *****.. *.* ..**.. **..*  ..************.******
MutL     204   YEEQGVHINREPFGPLTGTLVPPSMSNAVGITEALLAAEQGVKNITVGYGECGNMLQDIAALRCLEEQTNEYLKAYGYNDVFVTTVFHQWMGGFPQDESKAF

GlmL     306   GVIVTATTIAALAGATKVIVKTPHEAIGIPTKEANAAGIKATKMALNMLEGQRMPMSKELETEMAVIKAETKCILDKMFELGKGDLAIGTVKAFETGVMDIP
               ..*  ....*...******.*.****.****  *** *..*... ***.  *...*    .* *. .**.*.. .* *.****.**.* *** ***.**.*.*
GlmCA    305   AIISWGAAVAGMSGATKVITKSPHEAFGIPTAAANARGLRASRQMLNMVSDQKFPPCAAVEQEVDLIKSEVRAVLKKVFELGNGDVARGTVLAFEAGVLDVP
               ..*  ....*...******.*.****.****  *** *..*... ***.  *...*    .* *. .**.*.. .* *.****.**.* *** ***.**.*.*
MutL     306   GVIVTATTIASLAGATKVIVKTPHEAIGIPTKEANASGIKATKMALNMLEGQRMPMSKELETEMAIIKAETKCILDKMFELGKGDLAVGTVKAFETGVMDIP

GlmL     408   FGPSKYNAGKMMPVRDNLGCVRYLEFGNVPFTEEIKNYNRERLQERAKFEGRDVSFQMVIDDIFAVGKGRLIGRPE
               *.*.. ****..***** * .* ** *.**. ..*   ... . ***.****  *****.*** **....*****
GlmCA    407   FAPASCNAGKILPVRDNSGAIRVLEAGSVPLPKDILALHHDYVAERAHFEGRKPSFQMVVDDINAVSHSQLIGRP
               *.*.. ****..***** * .* ** *.**. ...   ... .****.****. *****.*** **....*****
MutL     408   FGPSKYNAGKMMPVRDNLGCVRYLEFGNVPFTEELKNYNRERLAERAKFEGREVSFQMVIDDIFAVGKGRLIGRPENK
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whole cells of the transformant, suggesting that the enzyme was overproduced in a soluble form. The strain could not grow in the medium under static or anaerobic conditions. On the other hand, the enzyme activity was not detected in the cells of the *E. coli* transformant harboring pMALCA3R, which contains a fragment in the opposite orientation to the *lac* promoter. The enzyme activity was also detected (11 000 units/l, 150 units/mg protein) in the cells of the recombinant strain grown in M9 medium containing glycerol and NH_4Cl, as carbon and nitrogen sources respectively, in the absence of (*S*)-glutamate.

The enzyme was purified in a crystalline form at a yield of 65.8% from the cell-free extracts of the transformant by the methods involving ammonium sulfate fractionation, Butyl-Toyopearl column chromatography, and crystallization (Table 1, Fig. 5). The specific activity of the purified enzyme was 235 units/mg and could be increased to 277 units/mg by dialysis against 10 mM buffer containing 0.5 mM EDTA, as in the case of wild-type MAL (Kato and Asano 1995b). This value was very close to that of wild-type MAL, which was 282 units/mg. There was no detectable difference in the apparent molecular mass between the recombinant and wild-type MAL (Fig. 5). Both enzymes had the same enzymological properties, such as substrate specificity, and effects of pH, temperature and inhibitors (data not shown). The N-terminal amino acid sequences of both enzymes were determined to be identical by automated Edman degradation (data not shown).

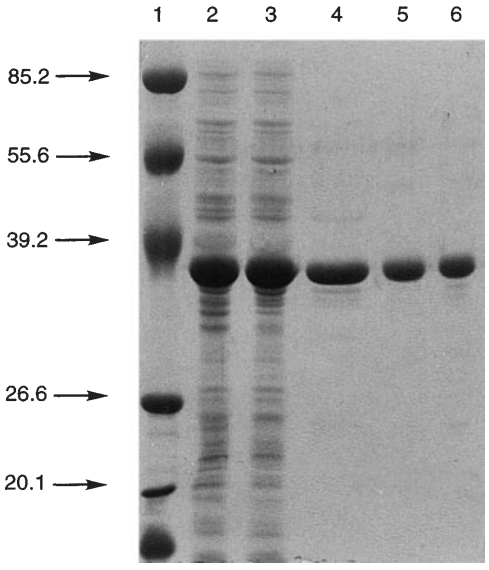

Fig. 5 Sodium dodecyl sulfate polyacrylamide slab-gel electrophoresis of the purified 3-methylaspartate ammonia-lyase from the recombinant *Escherichia coli* JM109/pMALCA3. Lanes: *1* the standard proteins, β-galactosidase (116 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triosephosphate isomerase (26.6 kDa), and trypsin inhibitor (21.1 kDa); *2* cell-free extract; *3* ammonium sulfate fractionation (40%–60%); *4* Butyl-Toyopearl fraction; *5* the crystalline enzyme; *6* the purified enzyme from *C. amalonaticus* strain YG-1002. Protein bands were stained with Coomassie brilliant blue R-250

Discussion

We show here the cloning, sequencing, and expression of MAL from a facultative anaerobic bacterium, *C. amalonaticus* strain YG-1002. The PCR reaction product (about 600 bp), amplified with oligonucleotides synthesized on the basis of the amino acid sequences of the N-terminal and internal peptides of the wild-type MAL, was used as a probe to clone the *mal* gene. The gene (1239 bp) encoded a polypeptide of 413 amino acid residues with an estimated molecular mass of 45 468 Da, which agreed with that of wild-type MAL (Kato and Asano 1995a). The deduced primary structure of MAL exhibited 71% similarity with MAL of the obligate anaerobe, *C. tetanomorphum*; however, we have reported differences in substrate specificities and kinetic properties between MAL from *C. amalonaticus* and *C. tetanomorphum* (Kato and Asano 1995a, b). It is interesting that some differences in the primary structure determine the enzymological properties of MAL. In the upstream and downstream regions of the *mal* gene, putative open reading frames, ORF1 and ORF2 respectively, could be identified. The deduced amino acid sequence of ORF1 showed high homology with those of the glutamate mutases of *C. amalonaticus* (Brecht et al. 1993) and *C. tetanomorphum* (Zelder et al. 1994). We previously reported the occurrence of glutamate mutase activity in the wild-type of *C. amalonaticus* strain YG-1002, and proposed the existence of a mesaconate pathway for (*S*)-glutamate fermentation in Enterobacteriaceae (Kato and Asano 1997). On the basis of these results, ORF1 can be assumed to code for a component of glutamate mutase and the existence of the mesaconate pathway in *Citrobacter* is also supposed. On the other hand, the role of the ORF2 product is not clear.

It is reported that some lyases, such as fumarase and aspartase, which catalyze an analogous reaction to that carried out by MAL, involving hydration and amination of fumaric acid (Hanson and Havir 1972) respectively, have a high degree of homology in their primary structure (Takagi et al. 1986). However, the primary

Table 1 Purification of 3-methylaspartate ammonia-lyase from the recombinant *E. coli* JM109/pMALCA3

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	1590	155000	97.8	100
Ammonium sulfate	1180	123000	104	79.0
Butyl-Toyopearl	477	109000	227	69.9
Crystallization	453	102000	235[a]	65.8

[a] This value could be increased to 277 (units/mg) by dialysis against 10 mM potassium phosphate buffer containing 0.5 mM 2-mercaptoethanol and 0.5 mM EDTA

structure of *Citrobacter* MAL showed no homology with those of the lyases. Recently the three-dimensional structure of aspartase from *E. coli* was determined by X-ray crystallographic analysis (Shi et al. 1997). Although MAL and aspartase are not similar in primary structure, it is expected that the two enzymes have a similar higher-order structure and reaction mechanisms. Comparison of the structures of these enzymes should elucidate the mechanism and evolutionary relationship among the amino acid ammonia-lyases. For higher-order structural elucidation of MAL, X-ray crystallographic studies are in progress.

Histidine and phenylalanine ammonia-lyases promote the first step in histidine degradation in most cells and the biosynthesis of lignins, flavonoids, and coumarins in plants (Hanson and Havir 1972), and are reported to contain the rare prosthetic group, dehydroalanine. It was recently shown that both enzymes make use of a Friedel-Crafts-type reaction, which was formerly thought to occur only in rather abiotic conditions (Rétey 1996). Like these enzymes, it has been proposed that *Clostridium* MAL contains dehydroalanine near its active site, since the enzyme was inhibited by various nucleophilic reagents (Botting and Gani 1992; Goda et al. 1992; Archer and Gani 1993), but the serine residue, which must be posttranslationally modified to dehydroalanine, has not yet been determined. However, these compounds did not inhibit MAL from the recombinant strain nor that from the wild-type strain (Kato and Asano 1995a, b). Site-directed mutagenesis experiments with serine residues of *Citrobacter* MAL would clarify the existence of the dehydroalanine residue in MAL.

The expression systems for MAL of the wild-type and recombinant strains were completely different. Wild-type MAL was produced only when the wild-type strain was grown statically or anaerobically in medium containing (*S*)-glutamate (Asano and Kato 1994a); however, the cells of the recombinant *E. coli* JM109/pMALCA3, in which the expression of the enzyme was controlled by the *lac* promoter on the vector, exhibited MAL activity well whenever they were grown aerobically in LB medium. The enzyme activity was also detected in the recombinant cells grown in the M9 medium, which did not contain (*S*)-glutamate. Since the genes for glutamate mutase and MAL are translationally coupled in the genome of *C. amalonaticus* strain YG-1002, both enzyme genes may be cotranscribed as a single operon in the strain, and expression of MAL might be controlled together with glutamate mutase expression. The production of GLM was also controlled by aeration and medium composition in the wild-type strain, as in the case of MAL (Kato and Asano 1997). This can be explained by the idea that both genes are cotranscribed by a single mRNA, the synthesis of which is induced by (*S*)-glutamate under anaerobic conditions. Further sequencing of the upstream region of ORF1 should elucidate the expression system of glutamate mutase and MAL, and the physiological function of the mesaconate pathway in Enterobacteriaceae.

Under optimized conditions, the MAL activity of *E. coli* JM109/pMALCA3 was about 50- and 20-fold more, per liter of culture and per milligram of protein respectively, than that of the wild-type strain (Kato and Asano 1997). The enzyme appeared to be soluble and in an active form in the cells, comprising over 40% of the total extractable cellular protein. From the cell-free extract of the transformant, the enzyme could be purified to homogeneity via three steps in high yield. In various enzymological properties, the recombinant enzyme was identical to the wild-type.

Since the enzyme can be produced in abundance by using a small amount of IPTG under aerobic conditions in medium without (*S*)-glutamate, we have started synthesizing a wide variety of substituted (*S*)-aspartates, containing two chiral centers, from commercially available fumarate derivatives using MAL.

References

Archer CH, Gani D (1993) Kinetics and mechanism of *syn*-elimination of ammonia from (2*S*, 3*R*)-3-methylaspartic acid by β-methylaspartase. J Chem Soc Chem Commun 140–142

Asano Y, Kato Y (1994a) Occurrence of 3-methylaspartate ammonia-lyase in facultative anaerobes and their application to synthesis of 3-substituted (*S*)-aspartic acids. Biosci Biotechnol Biochem 58: 223–224

Asano Y, Kato Y (1994b) Crystalline 3-methylaspartase from a facultative anaerobe, *Escherichia coli* strain YG-1002. FEMS Microbiol Lett 118: 255–258

Botting NP, Cohen MA, Akhtar M, Gani D (1988) Primary deuterium isotope effects for the 3-methylaspartase-catalyzed deamination of (2*S*)-aspartic acid, (2*S*, 3*S*)-3-methylaspartic acid, and (2*S*, 3*S*)-3-ethylaspartic acid. Biochemistry 27: 2956–2959

Botting NP, Gani D (1992) Mechanism of C-3 hydrogen exchange and the elimination of ammonia in the 3-methylaspartate ammonia-lyase reaction. Biochemistry 31: 1509–1520

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brecht M, Kellermann J, Plückthun A (1993) Cloning and sequencing of glutamate mutase component E from *Clostridium tetanomorphum*. FEBS Lett 319: 84–89

Dairi T, Asano Y (1995) Cloning, nucleotide sequencing, and expression of an opine dehydrogenase gene from *Arthrobacter* sp. strain 1 C. Appl Environ Microbiol 61: 3169–3171

Davis BJ (1964) Disk electrophoresis. II. Methods and application to human serum proteins. Ann New York Acad Sci 121: 404–427

Frank RL, and Vodkin LO (1991) Sequence and structure of a phenylalanine ammonia-lyase gene from *Glycine max*. DNA Sequence 1: 335–346.

Goda SK, Minton NP, Botting NP, Gani D (1992) Cloning, sequencing, and expression in *Escherichia coli* of the *Clostridium tetanomorphum* gene encoding β-methylaspartase and characterization the recombinant protein. Biochemistry 31: 10 747–10 756

Hanson K, Havir EA (1972) The enzymic elimination of ammonia. In: Boyer PD (ed.) The enzymes, 3rd edn, vol 7. Academic Press, New York, pp 75–164

Kato Y, Asano Y (1995a) Purification and properties of crystalline 3-methylaspartase from two facultative anaerobes, *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601. Biosci Biotechnol Biochem 59: 93–99

Kato Y, Asano Y (1995b) 3-Methylaspartate ammonia-lyase from a facultative anaerobe strain YG-1002. Appl Microbiol Biotechnol 43: 901–907

Kato Y, Asano Y (1997) 3-Methylaspartate ammonia-lyase as a marker enzyme of the mesaconate pathway for (*S*)-glutamate fermentation in *Enterobacteriaceae*. Arch Microbiol 168: 457–463

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Mikulskis AV, Cornelis GR (1994) A new class of proteins regulating gene expression in enterobacteria. Mol Microbiol 11: 77–86

Rétey J (1996) Enzymatic catalysis by Friedel-Crafts-type reactions. Naturewissenschaften 83: 439–447

Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 72: 619–629

Sanger FS, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Shi W, Dunbar J, Jayasekera MMK, Viola RE, Farber GK (1997) The structure of L-aspartate ammonia-lyase from *Escherichia coli*. Biochemistry 36: 9136–9144

Takagi JS, Tokushige M, Shimura Y, Kanehisa M (1986) L-Aspartate ammonia-lyase and fumarate hydratase share extensive sequence homology. Biochem Biophys Res Commun 138: 568–572

Wu PC, Kroening TA, White PJ, Kendrick KE (1992) Purification of histidase from *Streptomyces griseus* and nucleotide sequence of the *hutH* structural gene. J Bacteriol 174: 1647–1655

Zelder O, Beatrix B, Leutbecher U, Buckel W (1994) Characterization of the coenzyme-B_{12}-dependent glutamate mutase from *Clostridium cochlearium* produced in *Escherichia coli*. Eur J Biochem 226: 577–585