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Properties of Glutamate Racemase from *Bacillus subtilis* IFO 3336 Producing Poly-γ-Glutamate[1]

Makoto Ashiuchi,* Kazuhiko Tani,† Kenji Soda,‡ and Haruo Misono*,†,2

*Research Institute of Molecular Genetics and †Department of Bioresources Science, Kochi University, Nankoku, Kochi 783-8502; and ‡Faculty of Engineering, Kansai University, Suita, Osaka 564-0073

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We found glutamate racemase activity in cell extracts of *Bacillus subtilis* IFO 3336, which abundantly produces poly-γ-glutamate. The highest activity was obtained in the early stationary phase of growth. The racemase was purified to homogeneity. The enzyme was a monomer with a molecular mass of about 30 kDa and required no cofactor. It almost exclusively catalyzed the racemization of glutamate; other amino acids, including alanine and aspartate but not homocysteinesulfinate, were inactive as either substrates or inhibitors. Although the V_{max} value of the enzyme for L-glutamate is 21-fold higher than that for D-glutamate, the V_{max}/K_m value for L-glutamate is almost equal to that for the D-enantiomer. The racemase gene, *glr*, was cloned into *Escherichia coli* cells and sequenced. The racemase was overproduced in the soluble fraction of the *E. coli* clone cells with the substitution of ATG for TTG, the initial codon of the *glr* gene. D-Amino acid aminotransferase activity was not detected in *Bacillus subtilis* IFO 3336 cells. *B. subtilis* CU741, a *leuC7* derivative of *B. subtilis* 168, showed lower glutamate racemase activity and lower productivity of poly-γ-glutamate than *B. subtilis* IFO 3336. These results suggest that the glutamate racemase is mainly concerned in D-glutamate synthesis for poly-γ-glutamate production in *B. subtilis* IFO 3336.

Key words: glutamate racemase, *Bacillus subtilis*, poly-γ-glutamate producer, poly-γ-glutamate.

Several strains of *Bacillus* extracellularly produce poly-γ-glutamate in large quantities (*1-5*) in the stationary phase (*2, 6, 7*). The polymer usually consists of 70 to 80% D- and 20 to 30% L-glutamate (*6, 7*). Cells of *Bacillus* poly-γ-glutamate producers must produce one or more enzymes catalyzing the synthesis of a large amount of D-glutamate. Thorne *et al.* (*8*) suggested that D-glutamate was formed from L-glutamate through coupling of the D-amino acid aminotransferase reaction with the alanine racemase reaction in *Bacillus subtilis* ATCC 9945. However, Liu *et al.* (*9*) reported that D-amino acid aminotransferase activity was not found in cell extracts of *Bacillus pumilus*, whose characteristics are very close to those of *B. subtilis* (*10*). To elucidate the synthetic pathway of D-glutamate in *B. subtilis* cells, we examined the activities of both glutamate racemase and D-amino acid aminotransferase in cell extracts of *B. subtilis* IFO 3336 (formerly *Bacillus natto*, *B. subtilis* var. *natto*), a potent producer of poly-γ-glutamate, which is industrially used for the production of "natto," a traditional Japanese fermented food made from soybeans, and found high glutamate racemase activity and little D-amino acid aminotransferase activity in the cell extracts.

We describe the purification and characterization of glutamate racemase from *B. subtilis* IFO 3336 (*B. natto*), cloning of the enzyme gene into *Escherichia coli* cells, and overproduction of the gene product. We further discuss the physiological function of the enzyme.

MATERIALS AND METHODS

Materials—The DNAs of both the probe and primers for PCR were obtained from Hokkaido System Science, Hokkaido. pUC19, β-agarase, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and SeaPlaque GTG low-melt agarose were purchased from Takara Shuzo, Kyoto. The restriction enzyme, *Tsp*EI, was obtained from Toyobo, Osaka. L-Glutamate dehydrogenase (GDH) was purchased from Boehringer Mannheim, Germany. Homocysteinesulfinate was obtained from Tocris Cookson, UK. All other chemicals were of analytical grade.

Bacteria—*B. subtilis* CU741 [*leuC7*, *trpC2*] (*11*) was a kind gift from Professor Teruo Tanaka of Tokai University, Shizuoka. *E. coli* JM109 was purchased from Takara Shuzo. Other bacteria were obtained from the Institute for Fermentation, Osaka.

Culture Conditions for B. subtilis IFO 3336—After a spore suspension of *B. subtilis* IFO 3336 had been prepared

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[2] To whom correspondence should be addressed. E-mail: hmisono@cc.kochi-u.ac.jp

Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; IPTG, isopropyl-β-D-thiogalactopyranoside; GDH, L-glutamate dehydrogenase; DIG, digoxigenin; NTCB, 2-nitro-5-thiocyanatobenzoate.

(*2*), a 0.1-ml portion of the suspension was inoculated into 5 liters of a growth medium (pH 7.0) comprising 1% glucose, 0.5% polypepton, 0.25% yeast extract, 0.5% NaCl, and 0.05% $MgSO_4 \cdot 7H_2O$. Cultures were grown at 30°C for 30 h.

Preparation of Cell Extracts of B. subtilis—Harvested cells (40 g, wet weight) of *B. subtilis* IFO 3336 were suspended in 20 ml of the standard buffer [0.1 M Tris-HCl buffer (pH 8.0) containing 0.2% 2-mercaptoethanol and 10% glycerol] supplemented with 0.1 mM phenylmethanesulfonyl fluoride, and then disrupted by sonication at 4°C. The cell debris was removed by centrifugation. The supernatant solution was dialyzed against 4 liters of the standard buffer (pH 8.0) at 4°C overnight. The dialyzed solution was used as the cell extract.

Enzyme and Protein Assays—Glutamate racemase was assayed at 37°C with GDH (*12*). The mixture (1.0 ml) comprised 100 μmol of Tris-HCl buffer (pH 8.0), 10 μmol of D-glutamate, 5 μmol of NAD^+, 10 units of GDH, and enzyme. The reaction was started by the addition of D-glutamate. The activity was estimated from the initial rate of formation of L-glutamate determined by following the increase in the absorbance at 340 nm with a Shimadzu UV-2200A spectrophotometer. One unit of the racemase was defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-glutamate per min. The activity was alternatively measured by determination of each antipode formed from D- or L-glutamate by HPLC with a chiral carrier as follows. The reaction mixture (0.1 ml) comprising 10 μmol of Tris-HCl (pH 8.0), 1 μmol of D- or 20 μmol of L-glutamate, and enzyme was incubated at 37°C for 1 min. The reaction was terminated by the addition of 8 μl of 12 M HCl. The mixture was neutralized by the addition of 16 μl of 6 M NaOH. After 5-fold dilution of the mixture with a 2 mM $CuSO_4$ solution, a 5-μl aliquot was withdrawn and then applied to a CHIRALPAK MA(+) column (4.6×50 mm, DAICEL, Tokyo) in a Shimadzu LC-10A HPLC system. The column was developed with 2 mM $CuSO_4$ solution at the flow rate of 1 ml/min, with monitoring of the absorbance at 235 nm. Standard curves were prepared by estimation of the peak areas. The kinetic constants of the racemase were estimated by this method. D-Amino acid aminotransferase was assayed by determination of D-glutamate produced from 10 mM α-ketoglutarate and 25 mM D-alanine according to the method of Tanizawa *et al.* (*13*) by HPLC.

Protein was determined by means of a protein assay kit (Bio-Rad, USA) with BSA as a standard. The concentrations of the purified enzyme were derived from A_{275}. The absorption coefficient ($A^{1\%}_{1cm}$ at 275 nm=8.1) of the racemase of *B. subtilis* was estimated by the method of Scopes (*14*).

Enzyme Purification—All the purification procedures were performed at 10°C with a Bio-Rad Bio-Logic chromatography system. Each cell extract (300 mg of protein) dialyzed against the standard buffer (pH 8.0) was loaded onto an Econo-Pac High Q anion-exchange column (volume, 15 ml; Bio-Rad) equilibrated with the standard buffer (pH 8.0). After the column had been washed with 300 ml of the buffer, the enzyme was eluted with a linear gradient (150 ml+150 ml) of NaCl (0-0.5 M) in the buffer at the flow rate of 1 ml/min. The active fractions were combined and dialyzed against 2,500-fold volumes of the standard buffer (pH 8.0) at 4°C overnight. The dialyzed enzyme solution (12 ml) was concentrated with Centriprep-10 and Centricon-10 concentrators (Amicon, USA). The enzyme solution (1 ml) was applied to a Bio-Scale DEAE 2 anion-exchange column (volume, 2 ml; Bio-Rad) equilibrated with the standard buffer (pH 8.0) containing 0.1 M NaCl. The enzyme was eluted with a linear gradient (50 ml+50 ml) of NaCl (0.1-0.5 M) in the standard buffer (pH 8.0) at the flow rate of 0.5 ml/min. The active fractions (4 ml) were dialyzed against the standard buffer (pH 8.0) at 4°C overnight and then concentrated to 0.2 ml with the above concentrators. The solution was passed through a Superose12 HR 10/30 gel filtration column (10×300 mm, Pharmacia) equilibrated with the standard buffer (pH 8.0) containing 0.1 M NaCl. The column was developed with 30 ml of the same buffer at the flow rate of 0.2 ml/min. The active fractions (1.6 ml) were dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 0.2% 2-mercaptoethanol and 10% glycerol at 4°C overnight. The enzyme solution was concentrated to 0.2 ml with the Centricon-10 concentrator and then put on a Bio-Scale CHT-1 hydroxyapatite column (volume, 2 ml; Bio-Rad) equilibrated with the dialysis buffer (pH 7.0). The column was washed with 8 ml of the buffer and then eluted with a linear gradient (12 ml+12 ml) of potassium phosphate buffer (0.01-0.5 M) at the flow rate of 0.2 ml/min. The active fractions were combined, and then dialyzed against 0.1 M Tris-HCl buffer (pH 7.0) containing 0.2% 2-mercaptoethanol and 10% glycerol at 4°C overnight. The enzyme solution (0.4 ml) was applied to a Mono Q HR 5/5 anion-exchange column (5×50 mm, Pharmacia) equilibrated with the dialysis buffer (pH 7.0). The enzyme was eluted with a linear gradient (10 ml+10 ml) of NaCl (0-0.5 M) in the buffer at the flow rate of 0.5 ml/min. The enzyme solution (0.4 ml) was dialyzed against the standard buffer (pH 8.0), and then concentrated to 80 μl.

Molecular Mass Determination—The molecular mass of the enzyme was determined with the Bio-Logic chromatography system on a Superose12 HR 10/30 gel filtration column (10×300 mm) equilibrated with the standard buffer (pH 8.0) containing 0.1 M NaCl. A calibration curve was obtained with the following proteins: alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12 kDa).

Determination of the N-Terminal Amino Acid Sequence—The N-terminal amino acid sequence of the enzyme was determined with an Applied Biosystems 492 protein sequencer by the method of Matsudaira (*15*).

Construction of a DNA Library of B. subtilis—Chromosomal DNA (20 μg) of *B. subtilis* IFO 3336 was prepared with an ISOPLANT kit (Nippon Gene, Tokyo), and then partially digested with *Tsp*EI. The digested fragments of 1.0 to 3.0 kb were extracted from a 1.0% low-melting agarose gel and purified with β-agarase. The DNA library was constructed with the plasmid, pUC19. The purified fragments were ligated into the *Eco*RI site. The recombinant DNAs were introduced into cells of *E. coli* JM109. The cells were cultured on a plate of LB medium (*16*) supplemented with 50 μg/ml ampicillin, 0.1 mM IPTG, and 40 μg/ml X-Gal. White colonies were collected as the library clones.

Cloning—An oligonucleotide probe was designed from the N-terminal amino acid sequence of the enzyme. The

sequence is 5′-ATGGA(A,G)CA(A,G)CCIAT(T,C,A)GGI-GTIAT(T,C,A)GA(T,C)-3′ (I stands for a modified nucleotide, inosine). The probe was modified with digoxigenin (DIG) by means of a DIG Oligonucleotide 3′-End Labeling kit (Boehringer Mannheim). The library clones were transferred to nitrocellulose filters with a RepliPlate Colony Pad (Takara Shuzo). The clones on the filters were lysed. The filters were neutralized, dried, baked, and then washed. The fixed DNAs were hybridized with the DIG-labeled probe and detected by means of a DIG Nucleic Acid Detection kit (Boehringer Mannheim). Clones which hybridized with the probe were collected as positive clones harboring the racemase gene. A DNA fragment (1.2 kb) containing the gene was obtained from a clone and subcloned into pTrc99A, an *E. coli* expression vector, downstream of the *trc* promoter. We designated the plasmid obtained as pBSGR1.

Sequencing—The nucleotide sequence of the gene was determined with a PRISM kit (Perkin Elmer, USA), and an Applied Biosystems 373A DNA sequencer.

Construction of an Overproducer of Glutamate Racemase—The glutamate racemase gene (*glr*) in plasmid pBSGR1 was amplified by PCR with primers PBGR-NF1, which contained a typical ribosome-binding sequence (AGGA, boldface type), a sequence designed to replace the initial TTG of the gene with ATG (underlined), and a *Nco*I restriction site, and PBGR-CR1, containing an *Eco*RI restriction site. The sequences of the two primers were 5′-GAGCCATGG**AGGA**TCCTATTCATGGAACAACCAA-TAGGAGTCATT-3′ (PBGR-NF1) and 5′-GAGGAATTCC-GGCAAAACAGAAAAAAAC-3′ (PBGR-CR1). The reaction mixture for PCR (100 μl) consisted of 8 μmol of Tris-HCl buffer (pH 8.3), 2 μmol of $(NH_4)_2SO_4$, 0.5 μmol of $MgCl_2$, 20 nmol of each dNTP, 2.5 units of TaqGold DNA polymerase (Perkin Elmer), 0.5 μg of plasmid pBSGR1 (as a template), and 100 pmol each of PBGR-NF1 and PBGR-CR1. The reaction mixture was heated at 94°C for 1 min (for denaturation), cooled rapidly to 55°C for 1 min (for annealing), and then incubated at 72°C for 4 min (for extension). The programmed temperature shift was repeated 30 times. The amplified DNA fragment (0.8 kb) was digested with both restriction enzymes *Nco*I and *Eco*RI, and then ligated into the *Nco*I-*Eco*RI site of pTrc99A. We named the constructed plasmid pBSGR2. The nucleotide sequence of the racemase gene cloned in pBSGR2 was verified with the above DNA sequencer.

Isolation of Cloned Glutamate Racemase—Cells of the *E. coli* clone harboring pBSGR2, an overproducer of the enzyme, were inoculated into 1 liter of Luria broth medium containing ampicillin (50 μg/ml) and IPTG (2 mM) to induce enzyme production. The culture was carried out at 37°C for 18 h. Cells (5.0 g, wet weight) were suspended in 5 ml of the standard buffer (pH 8.0) supplemented with 0.1 mM phenylmethanesulfonyl fluoride, and then disrupted by sonication at 4°C. The cell debris was removed by centrifugation, and the supernatant solution was dialyzed against 1 liter of the standard buffer (pH 8.0) at 4°C overnight. The enzyme solution (5.3 ml, 9.2 mg/ml of protein concentration) was used as the cell extract. The cloned enzyme was isolated with the Bio-Logic chromatography system with Econo-Pac High Q anion-exchange, Bio-Scale CHT-1 hydroxyapatite, and Superose12 HR 10/30 gel filtration columns, according to the procedures for the enzyme purification from *B. subtilis* IFO 3336. The purified enzyme was concentrated up to approximately 10 mg/ml and stored at 4°C. The N-terminal amino acid sequence of the cloned enzyme was verified with the above protein sequencer.

Polyglutamate Determination—*B. subtilis* was grown in 200 ml of the growth medium under the above conditions, and the culture fluid was collected and filtrated. Poly-γ-glutamate in the culture fluid was isolated by the method of Kunioka (*2*). The resulting solution was incubated with Takara proteinase K (100 μg/ml) at 37°C for 2 h to remove α-polypeptides. The solution (2 ml) was dialyzed against water at 4°C overnight. A 10-μl portion of the solution was subjected to a SDS-PAGE (5% acrylamide). Poly-γ-glutamate was visualized by methylene blue staining according to the method of Ito *et al.* (*6*).

Nucleotide Sequence Accession Number—The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide databases under the following accession number, AB003685.

RESULTS

Glutamate Racemase Activities of Several Bacteria—We examined the glutamate racemase activities of several bacteria by the GDH coupling method, and found the activity in cell extracts of aerobic bacteria, *B. subtilis* IFO 3336 and CU741 (Table I). The activity of *B. subtilis* IFO 3336 is higher than those of *Lactobacillus fermentum* IFO 15885 and *Pediococcus pentosaceus* IFO 3182.

Glutamate Racemase and D-Amino Acid Aminotransferase Activities of B. subtilis—We assayed glutamate racemase and D-amino acid aminotransferase in cell extracts of *B. subtilis* IFO 3336 by HPLC. The racemization of L-glutamate increased with an increase in the incubation time. Although alanine racemase activity was found in the cell extract, little D-amino acid aminotransferase activity was detected under the conditions used. We measured both the glutamate racemase and D-amino acid aminotransferase activities in cell extracts of *B. subtilis* IFO 3336 obtained at each growth phase. The racemase activity was highest in cells in the early stationary phase, whereas little D-amino acid aminotransferase activity was found throughout growth. The racemase activity did not increase on the addition of 3% L- or D-glutamate to the medium.

TABLE I. **Glutamate racemase activity in cell extracts of several bacteria.**

Bacteria[a]	Glutamate racemase activity[b] (milliunits/mg)
Lactobacillus fermentum IFO 15885	18
Pediococcus pentosaceus IFO 3182	20
Clostridium butylicum IFO 13949	ND[c]
Staphylococcus aureus IFO 3060	ND[c]
Bacillus stearothermophilus IFO 12550	ND[c]
Bacillus subtilis IFO 3336 (*Bacillus natto*)	54
Bacillus subtilis CU741	8.2
Escherichia coli JM109	ND[c]
Salmonella typhimurium IFO 14212	ND[c]
Pseudomonas putida IFO 14162	ND[c]

[a]Bacteria were grown according to the manufacturer's instructions. [b]The activity was assayed by the GDH coupling method. Up to 50 μunits of the racemase activity could be detected with this assay method. [c]ND, not detectable.

Purification of Glutamate Racemase—The racemase was purified to homogeneity from cell extracts of *B. subtilis* IFO 3336. A summary of the purification is presented in Table II. The properties of the racemase described below were examined using the purified enzyme.

Molecular Mass and N-Terminal Amino Acid Sequence—SDS-PAGE gave a single band corresponding to a molecular mass of 30 kDa (Fig. 1), and the molecular mass was estimated to be 30 kDa by gel filtration on a Superose12 column (Fig. 2), showing that the enzyme is a monomer. The N-terminal 12 amino acid sequence was MEQPIGVIDSGV.

Stability—The enzyme was fully stable up to 37°C, and retained 90% of its activity at 50°C when heated for 10 min in 0.1 M Tris-HCl buffer (pH 8.0) containing 10% glycerol. The enzyme was most stable in the pH range of 6.0 to 10.5, when kept at 50°C for 10 min in the following buffers (0.1 M) containing 10% glycerol: sodium acetate buffer (pH 4.0-6.0), potassium phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 7.5-9.0), and *N*-cyclohexyl-3-aminopropanesulfonic acid buffer (pH 9.0-12.0).

Optimum pH and Temperature—The enzyme showed maximum activity at about pH 8.0 and significant activity over the pH range of 7.0 to 10.0. The optimum temperature for the enzyme activity was 37°C.

Cofactors—The absorption spectrum of the enzyme showed a maximum at 275 nm. No absorption peak was detected in the region from 300 to 700 nm. The enzyme was not affected by the addition of EDTA (10 mM), sodium borohydride, pyridoxal 5′-phosphate, FAD, NAD^+, $NADP^+$, ATP, $MgCl_2$, or $MnCl_2$ (1 mM each). Thus, the enzyme requires no cofactor.

Substrate Specificity—The enzyme exclusively acts on D- and L-glutamate. Both enantiomers of alanine, asparagine, aspartate, glutamine, lysine, proline, serine, and α-aminoadipate were inactive as substrates, and did not affect the glutamate racemization. Homocysteinesulfinate was a poor substrate.

Kinetics—When the racemization of D- and L-glutamate (10 mM each) was determined by HPLC, the apparent velocity of formation of D-glutamate from the L-enantiomer (203 μmol·min^{-1}·mg^{-1}) was 4.5 times higher than that of the reverse reaction (45 μmol·min^{-1}·mg^{-1}). Although the K_m and V_{max} values for D- and L-glutamate are different, as shown in Table III, the V_{max}/K_m values for D- and L- glutamate are similar. The K value of the enzyme reaction is nearly one. The K_m and V_{max} values for D- and L-homocysteinesulfinate are also different, but the V_{max}/K_m values for D- and L-homocysteinesulfinate are the same.

Effect of 2-Nitro-5-Thiocyanatobenzoate—The enzyme was inactivated on incubation with 0.2 mM 2-nitro-5-thiocyanatobenzoate (NTCB) at 37°C for 10 min. The enzyme activity was restored by incubation with 2 mM dithiothreitol at 37°C for 10 min. The addition of 20 mM D-glutamate protected the enzyme from the inactivation by NTCB.

Cloning and Nucleotide Sequence of the Glutamate Racemase Gene—We cloned the racemase gene into *E. coli* JM109 cells and sequenced the gene to determine the primary structure of the racemase. The gene encodes a protein consisting of 271 amino acid residues (Fig. 3). The predicted molecular mass (29,866 Da) is in good agreement

TABLE II. **Purification of glutamate racemase from *B. subtilis* IFO 3336.**

Steps	Total protein (mg)	Total units	Specific activity[a] (units/mg)	Yield (%)
Cell extract	300	16.2	0.054	100
Econo-Pac High Q	50	11.4	0.23	70
Bio-Scale DEAE 2	16	9.0	0.56	55
Superose12	1.8	8.0	4.4	49
Bio-Scale CHT-1	0.4	3.6	9.0	22
Mono Q	0.064[b]	2.7	42	17

[a]The activity was assayed by the GDH coupling method. [b]The concentration of the purified enzyme obtained in the MonoQ step was determined from A_{275} using the absorption coefficient ($A^{1\%}_{1cm}$ at 275 nm=8.1).

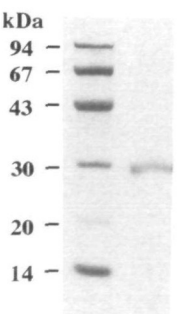

Fig. 1. **SDS-PAGE of the glutamate racemase purified from *B. subtilis* IFO 3336.** The enzyme (10 μg of protein) was subjected to SDS-PAGE (12.5% acrylamide) with the following molecular mass marker proteins: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

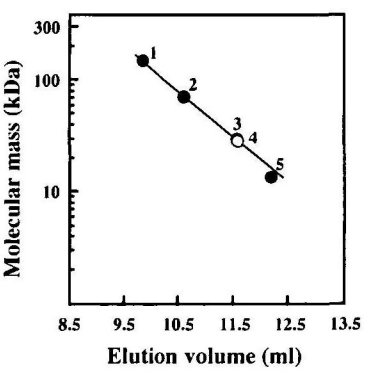

Fig. 2. **Estimation of the molecular mass of the glutamate racemase.** The molecular mass was estimated by the gel filtration method on a Superose12 column: 1, alcohol dehydrogenase; 2, bovine serum albumin; 3, carbonic anhydrase; 4, glutamate racemase; and 5, cytochrome *c*.

TABLE III. **Kinetic constants of the enzyme reactions.**

	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/mM)
D-Glutamate[a]	2.5	56	22.5
L-Glutamate[a]	50	1,150	22.4
D-Homocysteinesulfinate[a]	10	2.0	0.2
L-Homocysteinesulfinate[a]	200	40	0.2

[a]The enzyme activity was measured by determination of the antipode formed from the substrate by HPLC with a chiral carrier.

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taatcgaccgcatgcaaaaaaatgagcttgtggagcgggtcaaggacccggcggacagacgtgttgtccggatacatct

gctgcctgaaggagaacggattatccaagaggtcattacaaaaagacaggaatatctgctggatatgttcgaatcgttt

acagatgaagaaatagccatcttcgaaaaatctttgatgaaactgcagcatgaaatgaagagaaaatgaggcgattttg

ttg gaa caa cca ata gga gtc att gat tcc ggg gtt ggc ggt tta acc gtt gcg aag gaa
 M   E   Q   P   I   G   V   I   D   S   G   V   G   G   L   T   V   A   K   E

atc atg aga cag cta cct aaa gaa aat att atc tac gtc ggg gat acg aaa cgg tgt cct
 I   M   R   Q   L   P   K   E   N   I   I   Y   V   G   D   T   K   R   C   P

tat ggt ccg cgc cct gaa gaa gag gtg ctt caa tat acg tgg gag ctg acg aat tat tta
 Y   G   P   R   P   E   E   E   V   L   Q   Y   T   W   E   L   T   N   Y   L

ctc gaa aac cac cac atc aaa atg ctc gtg atc gcc tgt aat aca gca aca gcg atc gct
 L   E   N   H   H   I   K   M   L   V   I   A   C   N   T   A   T   A   I   A

ttg gat gac atc cag cgc agc gtc ggc ata ccg gtg gtc gga gtc atc cag cct ggt gcg
 L   D   D   I   Q   R   S   V   G   I   P   V   V   G   V   I   Q   P   G   A

aga gca gcg ata aaa gtg acg gat aat cag cat atc ggt gtc atc ggc aca gag aat acg
 R   A   A   I   K   V   T   D   N   Q   H   I   G   V   I   G   T   E   N   T

att aag agc aat gca tac gaa gaa gcg ctt ttg gca tta aac cct gat ttg aag gtt gaa
 I   K   S   N   A   Y   E   E   A   L   L   A   L   N   P   D   L   K   V   E

aac ctt gcc tgc ccg ctg ctt gtg cct ttt gtg gaa agc ggg aag ttt ctc gac aaa aca
 N   L   A   C   P   L   L   V   P   F   V   E   S   G   K   F   L   D   K   T

gca gac gag att gtt aaa acc tcg ctg tat ccg tta aaa gac aca tca att gat tcg ctg
 A   D   E   I   V   K   T   S   L   Y   P   L   K   D   T   S   I   D   S   L

att tta ggc tgc acc cat tac cct att tta aaa gaa gcc att caa aga tat atg gga gag
 I   L   G   C   T   H   Y   P   I   L   K   E   A   I   Q   R   Y   M   G   E

cac gta aac att att tcg tcc ggc gat gaa aca gcc cgg gaa gtc agc aca att ctc tct
 H   V   N   I   I   S   S   G   D   E   T   A   R   E   V   S   T   I   L   S

tat aaa ggg ctg ctg aac cag tct ccg att gcc ccg gat cat cag ttc ctg aca aca ggg
 Y   K   G   L   L   N   Q   S   P   I   A   P   D   H   Q   F   L   T   T   G

gcg cgt gat cag ttt gca aaa atc gca gac gat tgg ttt ggc cat gaa gtc ggg cat gtg
 A   R   D   Q   F   A   K   I   A   D   D   W   F   G   H   E   V   G   H   V

gaa tgt atc tca ctg caa gaa ccg att aaa aga tag tttttcccgcaacggtatgttgcgggttttt
 E   C   I   S   L   Q   E   P   I   K   R   *

ttctgttttgccggaattc
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Fig. 3. **Nucleotide sequence of the glutamate racemase gene and the deduced amino acid sequence of the enzyme.** The N-terminal amino acid sequence, that was determined by Edman degradation of the enzyme, is indicated by a single underline. The putative ribosome binding sequence is indicated by a double underline. Four different nucleotides in the reading frame from those of the *racE* gene (*30*) and one different amino acid residue in the enzyme from that of the RacE protein are shown as white letters.

with that of the enzyme isolated from *B. subtilis* IFO 3336. We designated the glutamate racemase gene as *glr*.

Overexpression of the glr Gene Encoding Glutamate Racemase of B. subtilis IFO 3336—The initial codon of the *glr* gene is TTG. The expression of the native *glr* gene was very low in *E. coli* JM109 cells (Fig. 4, lanes A and B). The specific activity of a cell extract of *E. coli* JM109/pBSGR1 was 1.0 milliunit·mg^{-1}. The initial TTG of the *glr* gene was replaced with ATG to produce a large amount of the gene product from the clone cells. A plasmid, pBSGR2, carrying the mutant gene was constructed and introduced into *E. coli* JM109 cells. The *glr* racemase was overproduced and mostly found in the soluble fraction of the clone cells (Fig. 4, lanes C and D).

Purification and Characterization of glr Racemase—The *glr* racemase was purified to homogeneity with a high yield compared with that from *B. subtilis* IFO 3336 cells (Table IV). The molecular mass of the enzyme was 30 kDa, and the N-terminal 10 amino acid sequence was determined to be MEQPIGVIDS. The enzymological and kinetic properties of the cloned enzyme were similar to those of the enzyme from *B. subtilis* IFO 3336.

Polyglutamate Productivity of B. subtilis—Poly-γ-glutamate was produced in a culture of *B. subtilis* IFO 3336 (Fig. 5, lane A). The polymer consisted mainly of D-glutamate (data not shown). *B. subtilis* CU741, which showed lower glutamate racemase activity, also produced the polymer in lower quantities than the IFO 3336 strain (Fig. 5, lane B). The molecular mass of the polymer produced by the CU741 strain (about 60 kDa) was lower than that of that produced by the IFO 3336 strain (more than 210 kDa).

DISCUSSION

We found glutamate racemase activity in cell extracts of *B. subtilis* IFO 3336, a poly-γ-glutamate producer. This

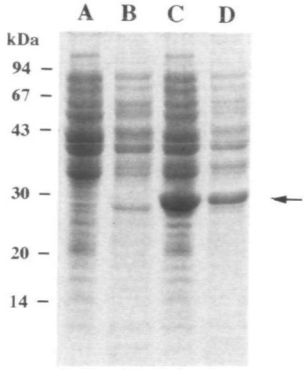

Fig. 4. **SDS-PAGE of cell extracts and cell pellet extracts of *E. coli* JM109/pBSGR1 and JM109/pBSGR2.** Cell extracts (50 μg of protein) and cell pellet extracts (30 μg) were subjected to SDS-PAGE (12.5% acrylamide). Cell pellet extracts were prepared as described previously (*35*). Lane A, cell extract of *E. coli* JM109/pBSGR1; lane B, cell pellet extract of *E. coli* JM109/pBSGR1; lane C, cell extract of *E. coli* JM109/pBSGR2; lane D, cell pellet extract of *E. coli* JM109/pBSGR2. The protein corresponding to the cloned glutamate racemase is indicated by an arrow.

TABLE IV. **Purification of glutamate racemase from the *E. coli* JM109 clone harboring pBSGR2.**

Steps	Total protein (mg)	Total units	Specific activity[a] (units/mg)	Yield (%)
Cell extract	50	215	4.3	100
Econo-Pac High Q	17	190	11	88
Bio-Scale CHT-1	7.0	175	25	81
Superose12	3.8[b]	160	42	74

[a]The activity was assayed by the GDH coupling method. [b]The concentration of the purified enzyme obtained in the Superose12 step was determined from A_{275} using the absorption coefficient ($A^{1\%}_{1cm}$ at 275 nm = 8.1).

activity has only been so far shown in lactic acid bacteria, facultative anaerobes, although the enzyme gene has been found in various bacteria (*9, 17-21*). Thus, this is the first report of the enzyme activity being found in cells of bacteria other than lactic acid bacteria. The enzymological properties of the glutamate racemase purified from *B. subtilis* IFO 3336, such as the optimum pH, cofactor independency, and substrate specificity, are similar to those of glutamate racemases from other bacteria (*9, 20, 22-25*). However, the enzyme was different from the others in kinetic parameters. The K_m and V_{max} values of the enzyme for L-glutamate are much higher than those for the D-enantiomer. In contrast, the K_m and V_{max} values of the racemases from *P. pentosaceus* (*24*), *E. coli* (*25*), and *Lactobacillus fermenti* (*20*) for both enantiomers of glutamate are closely similar to each other. It seems likely that the difference in the apparent reaction rate between the racemization of D- and L-glutamate is physiologically important: the enzyme can more rapidly convert a large amount of L-glutamate accumulated in cells to D-glutamate, and the conversion more effectively proceeds on coupling with a reaction that consumes D-glutamate such as poly-γ-glutamate synthesis (polymerization).

D-Glutamate has been thought to be produced through a D-amino acid aminotransferase reaction in *B. subtilis* cells

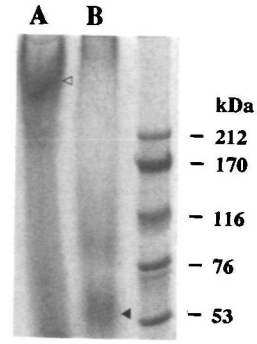

Fig. 5. **SDS-PAGE of poly-γ-glutamate in cultures of *B. subtilis* IFO 3336 and CU741.** Poly-γ-glutamate present in cultures (1 ml) of *B. subtilis* IFO 3336 (lane A) and CU741 (lane B) was electrophoresed as described under "MATERIALS AND METHODS" with standard protein markers: myosin (212 kDa), α_2-microglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa). Bands corresponding to the polymer produced mainly by *B. subtilis* IFO 3336 and CU741 are indicated by open and closed triangles, respectively.

(*8*). *B. subtilis* 168 shows significant D-amino acid aminotransferase activity (*26*). However, D-amino acid aminotransferase activity was not detected in cell extracts of *B. subtilis* IFO 3336. This indicates that glutamate racemase is the primary enzyme for D-glutamate synthesis in *B. subtilis* IFO 3336. The IFO 3336 strain produced poly-γ-glutamate in the culture medium. *B. subtilis* CU741, a *leuC7* derivative of *B. subtilis* 168, showed lower glutamate racemase activity and lower productivity of the polymer than the IFO 3336 strain; the geno- and phenotypes related to glutamate metabolism in the CU741 strain are essentially the same as those in the 168 strain (*27*). The polymer production by some strains of *B. subtilis* was stimulated by the addition of L-glutamate to the medium (*2, 3, 28, 29*). These results support that glutamate racemase producing D-glutamate directly from L-glutamate rather than D-amino acid aminotransferase plays an important role in D-glutamate synthesis for poly-γ-glutamate production.

The glutamate racemase gene, *glr*, was cloned and sequenced. Its sequence is highly homologous with that of the *racE* gene of *B. subtilis* 168 (*30*) (99.5% identity). The deduced amino acid sequence of the enzyme shows high similarity to those of other glutamate racemases (*9, 17-19, 31*). The overall homology scores of the racemase as to the racemases of *B. pumilus, P. pentosaceus*, and *E. coli* were estimated to be 73, 41, and 26%, respectively (Fig. 6). In particular, the regions surrounding the two cysteinyl residues (Cys-73 and Cys-184) are highly conserved; glutamate racemase reactions are proposed to proceed through a two-base mechanism (*23, 32*) involving the two essential cysteinyl residues (*9, 23, 31, 33*). The *B. subtilis* racemase was inactivated by NTCB, suggesting that the two conserved cysteinyl residues in the enzyme play an important role in the catalysis.

The *glr* racemase was overproduced in the soluble fraction of the *E. coli* clone cells with the substitution of ATG for TTG, the initial codon of the *glr* gene. The cloned enzyme showed similar properties to those of the racemase from *B. subtilis* IFO 3336, suggesting that the enzyme spontaneously and effectively folds to become active in the

Glr	(1-17)			MEQPIGV-	IDSGVGGLTV
B.p	(1-18)			MLDQPIGV-	IDSGVGGLTV
P.p	(1-18)			MDNRPIGF-	MDSGVGGLTV
E.c	(1-40)	MRQSMATKLQ	DGNTPCLAAT	PSEPRPTVLV	FDSGVGGLSV
	(18-57)	AKEIMRQLPK	ENIIYVGDTK	RCPYGPRPEE	EVLQYTWELT
	(19-58)	AKEIMRQLPK	EKIIYVGDTK	RCPYGPRKEE	EVLHYTNENA
	(19-58)	VKTAQKLLPN	EEIIFIGDEA	RMPYGPRPTA	EVVEFSRQMA
	(41-80)	YDEIRHLLPD	LHYIYAFDNV	AFPYGEKSEA	FIVERVVAIV
	(58-97)	NYLLENHHIK	MLVIACNTAT	AIALDDIQRS	VGIPVVGVIQ
	(59-98)	HYLLKHHHIK	MLVIACNTAT	AIALDEIKAY	LDIPVIGVIQ
	(59-97)	SFLMTKN-IK	ALVIACNTAT	NAALAVLQAE	LPIPVIGVIL
	(81-119)	TAVQERYPLA	LAVVACNTAS	TVSLPALREK	FDFPVVGVV-
			*		
	(98-135)	PGAR-AAIKV	TDNQHI-GVI	GTENTIKSNA	YEEALLALNP
	(99-136)	PGAR-YAIKV	YNNQHI-GVI	GYINTIKSEA	YKEALLSLKA
	(98-135)	PGAI-AANRQ	TKNQKI-GVI	ATLGTIKSEA	YPKALAEINT
	(120-157)	P-AIKPAARL	TANG-IVGLL	ATRGTVKRSY	THELIARFAN
	(136-174)	DLKVENLACP	LLVPFVESGK	FLDKTADEIV	KTSLY-PLKD
	(137-175)	GLTVQSLACP	LLVPFVESGT	FLDQTAEAVV	KDSLE-PMKE
	(136-174)	KLRAYPVACQ	EFVEIAEKNE	LHTTAAQKVM	NEKLA-EFRQ
	(158-197)	ECQIEMLGSA	EMVELAEAKL	HGEDVSLDAL	KRILRPWLRM
	(175-213)	TS-IDSLILG	CTHYPIL-KE	AIQRYMGEHV	NIISSGDETA
	(176-214)	TG-IDTLILG	CTHYPIL-KE	PIQRPMGSDV	SIISSGDETA
	(175-213)	DQ-IDTLILG	CTHFPLL-EE	GIQAAVGPDV	TLVDPGVETV
	(198-236)	KEPPDTVVLG	CTHFPLLQEE	LLQVLPEGTR	-LVDSGAAIA
			*		
	(214-253)	REVSTILSYK	GLLNQSPIAP	DHQFLTTGAR	DQFAKIADDW
	(215-254)	REASTILSYK	GLLNTSKEYP	VHTPYTTGQQ	QNPQNIARDW
	(214-253)	HQLIEILTKQ	ALQHAEGPKA	QDQYYSTGNI	KNFEEIARTF
	(237-276)	RRTAWLLEHE	APDAKSADAN	IAFCMAMTPG	AEQLLPVLQR
	(254-271)	FGHEVGHVEC	ISLQEPIKR		
	(255-272)	FGYLPGKVEY	VSLKHIYQQ		
	(254-265)	LNQDLRVEEV	KID		
	(277-289)	YGFETLEKLA	VLG		

Fig. 6. **Linear alignment of the amino acid sequences of the glutamate racemases of *B. subtilis* (Glr), *B. subtilis*, *B. pumilus* (B.p), *P. pentosaceus* (P.p), and *E. coli* (E.c).** Common residues in these racemases are shown in boldface type. The probable essential cysteinyl residues in catalysis are indicated by asterisks.

E. coli overproducer. In contrast, the glutamate racemases of *P. pentosaceus* (*34*) and *E. coli* (*35*) overproduced usually form inclusion bodies. Accordingly, it is difficult to obtain large amounts of these enzymes in native and active forms. This is of greater advantage than the production of the *P. pentosaceus* and *E. coli* racemases in inactive forms for studying the structure and function of glutamate racemase, and its applications.

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