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Cloning and Expression of the Glutamate Racemase Gene of *Bacillus pumilus*[1]

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A glutamate racemase gene (*murI*) was found in *Bacillus pumilus* cells and cloned into *Escherichia coli* WM335, a D-glutamate auxotroph, by means of a genetic complement method. *MurI* of *B. pumilus* encodes a 272-amino acid protein with an unusual initiation codon, TTG. The deduced amino acid sequence shows significant similarity with those of glutamate racemases from *E. coli* (ratio of identical residues, 28%), *Pediococcus pentosaceus* (44%), and *Staphylococcus haemolyticus* (49%). *B. pumilus* MurI was expressed as a fusion protein connected to the N-terminal 12 residues of β-galactosidase; the fusion protein showed glutamate racemase activity, and resembled the enzyme of *P. pentosaceus* in physicochemical and enzymological properties.

Key words: *Bacillus pumilus*, *murI*, glutamate racemase, purification, sequencing.

D-Glutamate is the common component of peptidoglycans of all eubacterial cell walls. D-Glutamate in a free form is incorporated into the common peptidoglycan precursor, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate (UDP-MurNAc-L-Ala-D-Glu), through the action of UDP-MurNAc-L-Ala-D-Glu synthetase [EC 6.3.2.9] (*17*). D-Glutamate is produced through two known routes: *i.e.* the reactions of D-amino acid aminotransferase [EC 2.6.1.21] (*11, 21, 23*) and glutamate racemase [EC 5.1.1.3] (*2-5, 7, 15*). The former catalyzes transamination between α-ketoglutarate and D-alanine, which is provided through the alanine racemase reaction. D-Amino acid aminotransferase has only been demonstrated in *Staphylococcus haemolyticus, Rhodospirillum rubrum*, and *Bacillus* strains. On the other hand, glutamate racemase activity has only been found in lactobacilli and *Pediococcus pentosaceus*. Thus, how other bacterial strains produce D-glutamate has been a puzzle.

Recently, however, a gene encoding glutamate racemase (*murI*) was found in *Escherichia coli* (*4, 5, 24*) and *S. haemolyticus* (*18*), respectively, and cloned. *MurI* compensates for the D-glutamic acid auxotrophy of a mutant *E. coli*, WM335, which has a nonsense mutation in *murI* (*12*). Thus, the expression of *murI* is indispensable for the growth of *E. coli* cells, although glutamate racemase activity has never been detected in extracts of cultured *E. coli* cells. Doublet *et al.* (*6*) found that *E. coli* glutamate racemase is activated about 100 times in the presence of UDP-MurNAc-L-Ala, the precursor of peptidoglycans. *E. coli* glutamate racemase is thus regulated post-translationally.

MurI of *S. haemolyticus* cloned into *E. coli* WM335 was expressed inefficiently, although a high-copy-number plasmid, pUC19, was used as a vector. Glutamate racemase activity in the extract of the transformant cells was only threefold higher than that in the control WM335 cells bearing only pUC19. UDP-MurNAc-L-Ala showed no effect on the enzyme activity of *S. haemolyticus* (*18*). Some unknown factors may activate the enzyme in *S. haemolyticus* cells; or an extremely small amount of D-glutamate may allow the growth of *S. haemolyticus* cells.

We have found that *Bacillus pumilus* is unusual as a *Bacillus* strain because it shows no D-amino acid aminotransferase activity. Although no glutamate racemase activity has been detected in cultured cells of *B. pumilus*, as in *E. coli* cells, we examined whether or not *murI* is also present in *B. pumilus* cells by means of PCR with primers designed on the basis of the common sequences in glutamate racemases of *E. coli* and lactobacilli. We found the occurrence of *murI*-like sequences in the genomic DNA of not only *B. pumilus* but also those of several other bacteria. We report here the cloning of *B. pumilus murI*, and the properties of the enzyme expressed as a fusion protein with β-galactosidase. We found that the initiation codon of the gene is TTG, which is attributable to the low expression of the gene in *B. pumilus* cells.

MATERIALS AND METHODS

Materials—*E. coli* WM335 strain (*leu pro his arg thyA met lac gal rpsL hsdM hsdR murI*), which requires D-glutamate for growth, was a kind gift from Professor Walter Messer of the Max-Planck Institute for Molecular Genetics, Germany. The *B. pumilus* strain used in this

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Abbreviations: UDP-MurNAc-L-Ala, UDP-*N*-acetylmuramoyl-L-alanine; IPTG, isopropyl-β-D-thiogalactopyranoside; DEAE, diethylaminoethyl; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

study was isolated in our laboratory, and identified by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Phagemid vectors pUC118 and pUC119, a shuttle vector, pHY300PLK, for *E. coli* and *B. subtilis*, helper phage M13KO7, *E. coli* JM109, *E. coli* BW313, *E. coli* BMH71-18 mutS, restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, T4 DNA kinase, and calf alkaline phosphatase were purchased from Takara Shuzo, Kyoto. A mixture of dNTPs was purchased from Boehringer Mannheim, Germany. L-Glutamate oxidase was a gift from Dr. H. Kusakabe of Yamasa Shoyu (Choshi). Other chemicals were of analytical grade.

Cloning of the Glutamate Racemase Gene of B. pumilus—Genomic DNA of *B. pumilus* was isolated, and then partially digested with 0.02 U of *Sau*3AI at 37°C for 10 min. Fragments (1.5-8.0 kbp) were isolated by agarose gel electrophoresis followed by electroelution (*20*), and then ligated into the *Bam*HI site of pUC118 at 12°C for 12 h with T4 ligase. A D-glutamate-dependent auxotroph, *E. coli* WM335, was directly transformed with the ligation mixture by electroporation. Two colonies grew on Luria-Bertani's (LB) medium containing ampicillin in the absence of added D-glutamate. Two plasmids, pGN1 and pGF1, containing 5- and 8-kbp insert DNA, respectively, were obtained from the transformant cells. The *Eco*RI-*Sac*I fragments (1.2- and 0.96-kbp) were isolated from pGN1 and pGF1, respectively, and then introduced into pUC118. The resultant plasmids were named pGN2 and pGF2, respectively. The plasmids were propagated in *E. coli* JM109 cells.

Enzyme and Protein Assays—Glutamate racemase was assayed by determination of the amount of L-glutamate formed from D-glutamate with L-glutamate oxidase. The standard assay mixture comprised 100 mM Tris-HCl buffer (pH 8.0), 10 mM D-glutamate, and the enzyme, in a final volume of 50 μl. The reaction was started by the addition of the enzyme, carried out at 37°C for 5-15 min, and stopped by boiling for 10 min. After centrifugation of the mixture, the supernatant was incubated at 37°C for 1 h with 100 mM Tris-HCl buffer (pH 8.0), 24 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, 24 mM 4-aminoantipyrine, 5 units of peroxidase, and 5 units of L-glutamate oxidase, in a final volume of 100 μl. The increase in absorbance at 555 nm was measured with a Beckman DU-50 spectrophotometer. Alternatively, glutamate racemase was assayed by determination of the amount of α-ketoglutarate formed from L-glutamate with 2,4-dinitrophenylhydrazine. The enzyme reaction was carried out under the same conditions as described above. The supernatant of the reaction mixture was incubated at 37°C for 1 h with 100 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, and 5 units of L-glutamate oxidase, in a final volume of 100 μl. The solution was mixed with 100 μl of 0.066% 2,4-dinitrophenylhydrazine dissolved in 2 M HCl, followed by the addition of 400 μl of 2 M NaOH. The increase in absorbance at 520 nm was measured. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-glutamate per min.

Protein concentrations were determined with a Bio-Rad protein assay kit with bovine serum albumin as a standard.

Purification of Fusion Glutamate Racemase—*E. coli* XL1-BLUE cells containing pGF2 were cultivated at 37°C for 2 h in 3.5 liters of LB medium containing 4% of glycerol and 50 μg/ml of ampicillin. After the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium, the cultivation was continued for a further 8 h. The cells were harvested by centrifugation and washed with 0.9% NaCl. The following purification procedures were carried out at 4°C. The cells (20 g wet weight) were disrupted by sonication for 20 min in Buffer I consisting of 0.1 M Tris-HCl buffer (pH 8.0), 10% glycerol, 1 mM DL-glutamic acid, 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonylfluoride, and 1 mM EDTA, and then centrifuged. The supernatant was fractionated by ammonium sulfate precipitation. The most active fraction (40-50% saturation) was dissolved in Buffer I containing 30%-saturated ammonium sulfate, and then applied to a Butyl-Toyopearl column (bed volume, 50 ml) equilibrated with the same buffer. After the column had been washed with 100 ml of the same buffer, the enzyme was eluted with a 300-ml linear gradient of 30-0% saturated ammonium sulfate in Buffer I. The active fractions were combined. The enzyme was precipitated with ammonium sulfate, and then dissolved in and dialyzed against Buffer I. The enzyme solution was then applied to a DEAE-Toyopearl column (bed volume, 20 ml) equilibrated with Buffer I. After the column had been washed with the same buffer, the enzyme was eluted with a 80-ml linear gradient of 0-0.4 M KCl in Buffer I. The active fractions were combined and concentrated with a Centricon-10. The purity of the enzyme was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Antiserum against the purified enzyme was prepared in a rabbit.

Molecular Weight Determination—The molecular weight of the native enzyme was determined by gel filtration on a Superose 12 HR 10/30 column (Pharmacia, Sweden) with 0.5 M potassium phosphate buffer (pH 7.5) as the mobile phase. The molecular weight was also determined by SDS-PAGE with 12.5% polyacrylamide.

N-Terminal Amino Acid Sequence Analysis—The purified glutamate racemase (about 100 pmol) was applied to a membrane support according to the method of Matsudaira (*13*). The membrane was subjected directly to automated Edman degradation with a Shimadzu PPSQ10-S protein sequencer.

DNA Sequencing of the Glutamate Racemase Gene—The cloned DNA fragments in pGN2 and pGF2 were sequenced by the dideoxy chain termination method with an Applied Biosystems Model 370A DNA sequencer (Perkin-Elmer, USA). The *murI* gene was also sequenced directly, with the chromosome of *B. pumilus* as a template, by means of a TaKaRa LA PCR *in vitro* Cloning Kit (Takara, Kyoto) according to the manufacturer's protocol. The genomic DNA of *B. pumilus* was digested with *Eco*RI, and the resultant fragments were ligated with an *Eco*RI cassette. The primers used were: Cassette primers C1 and C2 in the kit, and synthetic primers, S1 and S2, designed according to the sequence of *murI*: 5′-TGAACCGTTAGACCTGCTTTTAGTGAC-3′ (S1); 5′-AATATGGTGGTGTTTTAGTAAGTAGTGTGC-3′ (S2). The PCR reaction was carried out with DNA in the above ligation mixture as a template, and primers C1 and S1. The product was used for the second PCR reaction with C2 and S2 as primers. The second PCR product was purified and sequenced.

Site-Directed Mutagenesis—The putative initiation codon, TTG, of the cloned *murI* gene was replaced by ATG by the

Kunkel method (*10*). Single-stranded DNA for *murI* containing uracil was prepared with bacteriophage M13-KO7 from *E. coli* BW313 carrying pGN2, and used as the template. The primer was: 5′-GGTTGATCCAACATAAT-CGCC-3′. The resulting plasmid containing the mutant gene was confirmed by DNA sequencing and named pGA1.

Construction of a Shuttle Vector Coding for murI for Expression in B. subtilis—The *Eco*RI-*Hin*dIII fragments (1.2-kbp) were isolated from pGN2 and pGA1, and introduced into a shuttle vector, pHY300PLK (Yakult,

Fig. 1. **The partial nucleotide sequence of the fusion, *murI*, and the deduced amino acid sequence.** The nucleotide sequence of the upstream region of the open reading frame and that corresponding to the N-terminal 12 amino acid residues are the same as those of *lacZ* of the vector, pUC118. The following nucleotide sequence was compatible with that of the wild-type *murI*. The indicated 20-amino-acid sequence was confirmed by N-terminal amino acid sequencing. The *Sau*3AI site is underlined

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ATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCA
                                  -35                 -10
CTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC
          SD                                                    Sau3AI
ACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCAACCAATCGGCGTCATTGATTCC
                    M  T  M  I  T  N  S  S  S  V  P  G  D  Q  P  I  G  V  I  D...
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GCAGCACAACCACTGATTTAATTGATAGAATGGAAAAAAGTGAACTTGTCGAACGGGTGAAAGATCCATCTGACC      75

GCCGCGTGGTTCGCATTCATTTATTGCCTGAAGGCGAGCGGATCATTCAAGAAGTGATTGCTAAACGCCAAGAAT     150
                          -35                                 -10
ACGTCAGTGAACTTCTAGGGGCTTTCTCAGAAGAAGAAGCCATCGTTTTCAACCAATCCTTAACGAAACTACAGC     225
                     SD     +1
AGCAAATGAAAAGAAAATGAGGCGATTTTGTTGGATCAACCAATCGGCGTCATTGATTCCGGCGTCGGCGGTTTA     300
                                M  L  D  Q  P  I  G  V  I  D  S  G  V  G  G  L      16
ACCGTTGCAAAGGAAATCATGAGACAACTGCCAAAAGAAAAAATCATTTATGTAGGCGATACAAAACGATGTCCA     375
 T  V  A  K  E  I  M  R  Q  L  P  K  E  K  I  I  Y  V  G  D  T  K  R  C  P      41
                                                   S2
TATGGCCCGCGGAAAGAAGAAGAAGTTCTTCATTATACGTGGGAAATGGCACACTACTTACTAAAACACCACCAT     450
 Y  G  P  R  K  E  E  E  V  L  H  Y  T  W  E  M  A  H  Y  L  L  K  H  H  H      66

ATTAAAATGCTCGTCATTGCATGCAACACAGCAACGGCGATCGCACTGGATGAAATCAAAGCAACGCTTGATATT     525
 I  K  M  L  V  I  A  C  N  T  A  T  A  I  A  L  D  E  I  K  A  T  L  D  I      91

CCGGTCATTGGCGTCATTCAGCCTGGCGCCCGTACAGCGATCAAGGTGACAAACAACCAGCATATTGGCGTTATT     600
 P  V  I  G  V  I  Q  P  G  A  R  T  A  I  K  V  T  N  N  Q  H  I  G  V  I     116
                                                   S1
GGCACAATCAATACGATCAAAAGCGAGGCATACAAAGAAGCGCTGCTGTCACTAAAAGCAGGTCTAACGGTTCAA     675
 G  T  I  N  T  I  K  S  E  A  Y  K  E  A  L  L  S  L  K  A  G  L  T  V  Q     141

AGTCTGGCGTGTCCGCTCCTTGTTCCATTTGTGGAAAGCGGTACGTTTTTAGATCAGACAGCAGAGGCAGTGGTC     750
 S  L  A  C  P  L  L  V  P  F  V  E  S  G  T  F  L  D  Q  T  A  E  A  V  V     166

AAAGATTCCCTTGAACCGATGAAAGAAACAGGAATTGACACTCTTATTCTTGGCTGCACACACTATCCGATTTTA     825
 K  D  S  L  E  P  M  K  E  T  G  I  D  T  L  I  L  G  C  T  H  Y  P  I  L     191

AAAGAGCCGATTCAGCGCTTTATGGGCAGTGATGTCAGCATTATTTCATCAGGAGATGAGACAGCGAGAGAAGCA     900
 K  E  P  I  Q  R  F  M  G  S  D  V  S  I  I  S  S  G  D  E  T  A  R  E  A     216

AGTACGATTCTTTCATATAAAGGGCTGTTAAATACCTCTAAAGAAACGCCTGTCCATACATTTTACACAACAGGG     975
 S  T  I  L  S  Y  K  G  L  L  N  T  S  K  E  T  P  V  H  T  F  Y  T  T  G     241

CAGCAGCAAAATTTTCAAAACATCGCTCGTGACTGGTTTGGCTACCTGCCAGGGAAGGTCGAAACCGTGTCACTT    1050
 Q  Q  Q  N  F  Q  N  I  A  R  D  W  F  G  Y  L  P  G  K  V  E  T  V  S  L     266

GAACACATCTACCAGCAATAACCCGCTTCTATTTGAAGTGGGTTATTTTTTTATGAAAAGGGTCTAATCCTCTGG    1125
 E  H  I  Y  Q  Q  *                                                            272
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Fig. 2. **Nucleotide sequence of *B. pumilus murI* and the deduced amino acid sequence of glutamate racemase.** The −35 and −10 regions of the putative promoter are underlined. The possible Shine-Dalgarno sequence is double underlined, and the initiation codon, TTG, is single underlined. The *Sau*3AI site, and the positions of primers S1 and S2 used for the genomic DNA sequencing are also indicated, with a dashed underline.

Tokyo). The resultant plasmids, pGNS and pGAS, respectively, were propagated in *E. coli* C600, and then used for the transformation of *B. subtilis*.

RESULTS

Cloning of murI of B. pumilus—The genomic DNA of *B. pumilus* was partially digested with *Sau*3AI and then ligated into the *Bam*HI site of pUC118. The D-glutamate-requiring auxotroph, *E. coli* WM335, was transformed with the resulting plasmids in order to clone the *murI* gene, which was expected to allow the auxotroph to grow. Thus, we obtained two types of clone cells, from each of which we isolated a plasmid, pGN1 and pGF1. We digested them further to obtain plasmids named pGN2 and pGF2, respectively, as described under "MATERIALS AND METHODS." pGN2 was found to carry the natural *murI* derived from *B. pumilus*, although as described below, the gene was expressed at an extremely low level due to its unusual initiation codon, TTG. On the other hand, pGF2 was found to carry a fused gene comprising *lacZ* and *murI* connected to each other at the *Sau*3AI site, as shown in Fig. 1. A fused protein with high glutamate racemase activity was produced in a large quantity by *E. coli* clone cells carrying the plasmid, as described below.

Nucleotide Sequence of murI of B. pumilus—The DNA

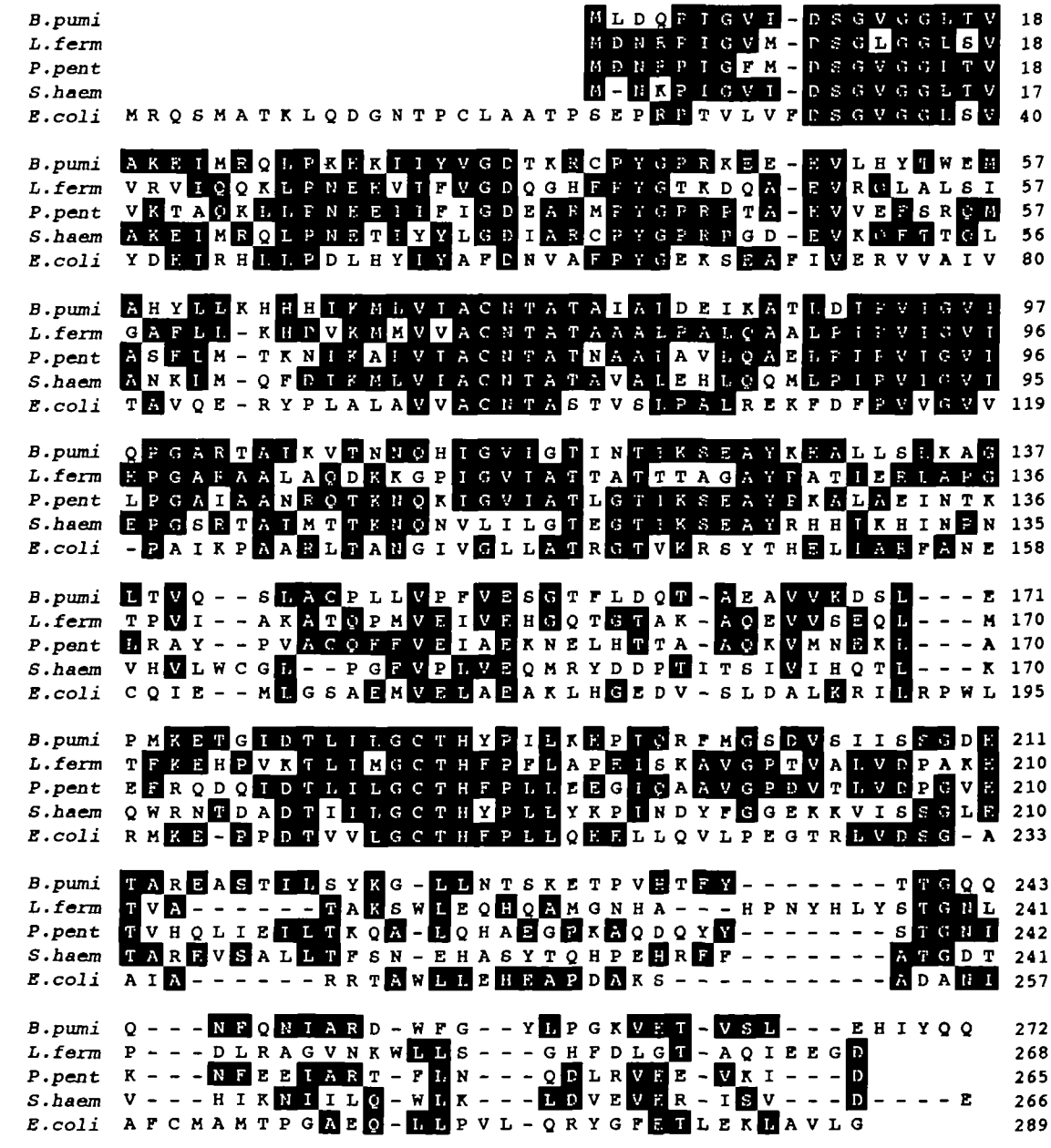

Fig. 3. **Alignment of the deduced amino acid sequence of glutamate racemase of *B. pumilus* (*B. pumi*) with those of *Lactobacillus fermenti* (*L. ferm*), *P. pentosaceus* (*P. pent*), *S. haemolyticus* (*S. haem*), and *E. coli* (*E. coli*).** Identical residues in the sequences are denoted by white letters in black boxes. The alignment was performed with the alignment software, MegAlign, using the Jotun Hein Method.

sequencing of *murI* revealed an ambiguous initiation codon (Fig. 2). However, if we assume that one of the three triplets, ATT-TTG-TTG, which occur downstream of the putative ribosome binding site, AAATGAGGCGA, and the possible promoter, TTAACGA (−10), together with TTC-TCA (−35), serve as the native initiation codon, then we can deduce the amino acid sequence of MurI, which is very similar to those of the same enzymes from other bacterial sources: *E. coli* (ratio of identical residues, 28%), *P. pentosaceus* (44%), and *S. haemolyticus* (49%) (Fig. 3).

In order to rule out the possibility that the unusual initiation codon was due to an artifact of our genetic manipulation, we determined the nucleotide sequence around the N-terminal region of the gene directly, with the genomic DNA as a template, by PCR. However, we found a sequence identical to that of the cloned gene. Both ATT and TTG are known to each serve as an initiation codon (*9, 14*), and any of the three triplets, ATT-TTG-TTG, could be the native initiation codon. Glutamate racemase was partially purified from a cell extract of *E. coli* JM109 carrying pGN2 by Butyl-Toyopearl column chromatography, although the enzyme was produced by the cells at a level barely detectable on immunoblot analysis with antiserum raised against the fusion enzyme (see below). The N-terminal sequence of the wild-type enzyme blotted onto a PVDF membrane was determined to be M-L-D-Q-I-G-V-I-D-S-G-. This indicates that the native initiation codon is the first TTG of ATT-TTG-TTG (Fig. 2).

Replacement of the Native Initiation Codon with ATG, and Expression of the Mutant Gene in E. coli and B. subtilis—We replaced the native initiation codon, TTG, with ATG by site-directed mutagenesis. The glutamate racemase activity of a cell extract of *E. coli* JM109 harboring the resulting plasmid named pGA1 was about three-times higher than that of that containing pGN2 encoding the wild-type gene. However, both of them showed essentially the same growth curve, as shown in Fig. 4. When *E. coli* WM335 was used as the host, the logarithmic phase of the ATG clone started after a lag phase, which was much longer than that in the case of the wild-type TTG clone (data not shown).

We introduced the mutant ATG gene into a shuttle vector, pHY300PLK, for expression in *B. subtilis*. No glutamate racemase activity was detected in a cell extract of *B. subtilis* containing the plasmid named pGAS, as in the extract of *B. subtilis* harboring pGNS derived from pHY300PLK and the native *murI*. However, immunoblot analyses of the cell extracts suggested that the mutant ATG gene was expressed much more than the wild-type gene (Fig. 5).

Properties of the Fused Glutamate Racemase—We purified the fused glutamate racemase to homogeneity from a cell extract of *E. coli* XL1-BLUE harboring pGF2 (Fig. 6). The results of the purification are shown in Table I. The N-terminal sequence of the purified enzyme was determined to be M-T-M-I-T-N-S-S-S-V-P-G-D-Q-P-I-G-V-I-D, which is identical with that deduced from the DNA sequence. The sequence of the underlined 12 residues is compatible with that of the N-terminus of β-galactosidase.

The molecular weight of the enzyme determined by SDS-PAGE was 31,000, which is similar to the value calculated from the deduced amino acid sequence (30,985). The molecular weight was also estimated to be about 31,000 by gel filtration. This suggests that the enzyme is a monomeric protein.

The enzyme activity was not affected by the addition of 10 mM of EDTA, hydroxylamine, or sodium borohydride.

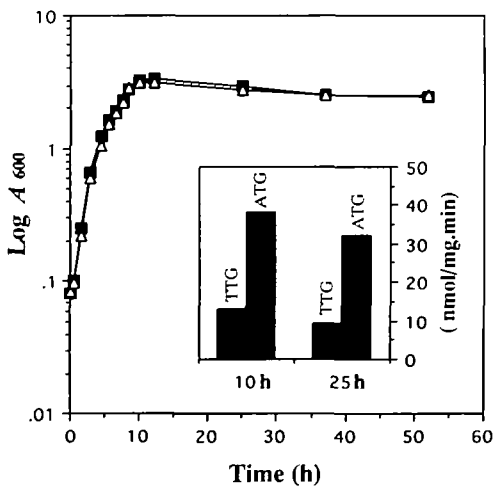

Fig. 4. **Growth curves of *E. coli* JM109 cells containing the plasmid, pGN2, encoding the wild-type *murI* (■), and ones containing the plasmid, pGA1, encoding the mutant *murI* with the ATG initiation codon (△), and the glutamate racemase activity of the cell extracts.** Cells were grown at 37°C in LB medium. A portion of the cells was harvested at 10 and 25 h after inoculation, washed and disrupted by sonication, followed by centrifugation. The glutamate racemase activity in the supernatant was assayed.

Fig. 5. **Immunoblot analysis of cell extracts of *B. subtilis* cells containing the plasmid, pGAS, encoding the mutant *murI* with the ATG initiation codon (Lane 1) and ones containing pGNS encoding the wild-type *murI* (Lane 2).** Transformant *B. subtilis* cells were cultured at 37°C for 14 h in LB medium. Cell extracts were prepared by the same method as that described in the legend to Fig. 4. The cell extracts (10 μg protein each) were subjected to SDS-PAGE.

TABLE I. **Summary of purification of the fusion glutamate racemase from *E. coli* XL1-BLUE/pGF2.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell extract	1,430	1,010	0.72	100
40-50% $(NH_4)_2SO_4$	798	608	0.77	60
Butyl-Toyopearl	55.0	346	6.3	34
DEAE-Toyopearl	15.0	287	19	28

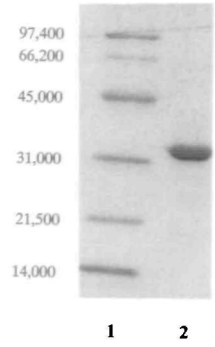

Fig. 6. **SDS-PAGE of the purified fusion glutamate racemase.** Lane 1, molecular weight markers; phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400), from top to bottom. Lane 2, purified fusion glutamate racemase.

The absorption spectrum of the enzyme showed no peaks in the near-UV or visible region except for the absorption due to tryptophan and tyrosine residues of the protein. These results suggest that the enzyme contains no cofactors such as nicotinamides, flavins or pyridoxal 5′-phosphate. The enzyme was completely inactivated by thiol reagents (1 mM each); *N*-ethylmaleimide and *p*-chloromercuribenzoate. The deduced amino acid sequence of the fusion protein indicates the occurrence of two cysteinyl residues (Cys74 and Cys185), which correspond to the essential cysteine residues of the *E. coli* enzyme (*6, 8, 24*).

Neither the homogeneous preparation of the fusion enzyme nor the partially purified preparation of the wild-type enzyme was activated by UDP-MurNAc-L-Ala: the *B. pumilus* enzyme is distinct from the *E. coli* enzyme, which is activated by the compound (*6, 8*). Thus, the *B. pumilus* enzyme is similar to the enzyme from other Gram-positive bacteria: *P. pentosaceus* and *Lactobacillus fermenti* (*3, 7, 22*).

DISCUSSION

This is the first report showing the occurrence of a glutamate racemase gene, *murI*, in *Bacillus* strains. The *Bacillus* MurI is similar to the enzymes from other bacterial sources: it contains essential cysteine residues. Both the two essential cysteine residues of the *E. coli* enzyme are aligned exactly as in the *B. pumilus* enzyme. Therefore, Cys74 and Cys185 of the *B. pumilus* enzyme probably participate in catalysis, in the same manner as in the *E. coli* and *P. pentosaceus* enzymes.

The *B. pumilus murI* uses TTG as an initiation codon. The same initiation codon is used by several *E. coli* genes, but only rarely. However, Gram-positive bacteria including bacilli use it much more frequently: about 17% of the total initiation codons examined for many genes of Gram-positive bacteria (*16*). Some genes with the TTG initiation codon are known to be expressed under particular regulation (*9, 19*). The expression of the *E. coli* adenylate cyclase gene containing TTG and ATG, respectively, as the initiation codon was compared in *E. coli* (*19*). The ATG gene was expressed at levels from 2 to 6 times higher than that of the TTG gene, and the growth conditions influenced their expression levels (*19*). Khudyakov *et al.* (*9*) also reported that ATG acts from 1.7 to 3.3 times more efficiently as an initiation codon than TTG when they are used in a *lacIZ* hybrid gene. The *B. pumilus murI* gene with the ATG initiation codon was expressed about 3 times more efficiently than that with TTG in *E. coli*. As we have shown here, ATG is also better as an initiation codon than TTG in *B. pumilus*.

Glutamate racemase activity has only been demonstrated in lactic acid bacteria, although *murI* genes have been found in several other bacterial strains. Baliko and Venetianer suggested the physiological importance of the attenuation of the *murI* expression (*1*). They showed that high expression of *murI* reduces the negative superhelicity of DNA and brings about the aberration in nucleoid separation during the cell division of *E. coli* (*1*). However, no difference was found between the growth curves of *E. coli* cells containing the wild-type *murI* and the mutant *murI* with the ATG initiation codon, respectively. Thus, the expression level of the *B. pumilus murI* does not affect the growth of *E. coli* cells. The cultured *B. pumilus* cells showed no glutamate racemase activity even though they had the *murI* gene. If we assume that D-glutamate is provided through the glutamate racemase reaction, then the *murI* expression is controlled in *B. pumilus* cells. Whatever the mechanism of control is, the initiation codon, TTG, is one of the factors suppressing the expression.

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