Purification, Cloning, and Cofactor Independence of Glutamate Racemase from *Lactobacillus*†

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ABSTRACT: Glutamate racemase has been purified more than 12 000-fold from *Lactobacillus fermenti*. The racemase gene has been cloned using standard hybridization techniques combined with a novel selection for in vivo glutamate racemase activity, and the racemase has been expressed in *Escherichia coli* as 20–25% of the total soluble cell protein. The cloned gene product is indistinguishable from that purified from *Lactobacillus* and is a monomer of M_r 28 300. Both a coupled enzymatic assay and a circular dichroism assay show that the enzyme follows Michaelis–Menten kinetics, with a K_m of 0.3 mM and a k_{cat} of 70 s^{-1} in each reaction direction. Investigations into the cofactor dependence of glutamate racemase indicate that the enzyme employs neither pyridoxal phosphate nor a pyruvoyl group in the labilization of the proton at the stereogenic center of glutamate. Furthermore, the racemase activity is unaffected by the presence of the metal-chelating reagent EDTA. The gene sequence of the racemase is 24% identical to that of aspartate racemase from *Streptococcus thermophilus* and 30% identical to that of an unidentified open reading frame in the *rrnB* ribosomal RNA operon of *E. coli*. Because the two cysteine residues in glutamate racemase and their surrounding regions are well-conserved in both of these sequences, and since glutamate racemase is stabilized by the presence of reduced thiols, these residues are possible candidates for the enzymic bases that deprotonate glutamate at C-2.

The racemization of α-amino acids occurs extremely slowly in the absence of catalysts. Under biological conditions (pH 7.6, 37 °C) the configurational half-lives of several α-amino acids have been estimated to range from 50 years (for serine) to 6500 years (for isoleucine) (Bada, 1984). This remarkable stability, which is similar to that of amino acid residues embedded in peptides, ensures the stereochemical integrity of proteins in vivo.[1]

Glutamate racemase (EC 5.1.1.3) catalyzes the interconversion of the enantiomers of glutamic acid. The enzyme is found in the lactic acid family of bacteria, notably in *Lactobacillus*, where it provides the (*R*)-glutamic acid that is presumably required for cell wall biosynthesis. (*R*)-Amino acids are, in fact, essential for the viability of many organisms, including all bacteria (with the possible exceptions of the methanogens and the extreme halophiles; Cummins, 1989). Besides the use of (*R*)-amino acids in the construction of the peptidoglycan of bacterial cell walls, the "unnatural" amino acid configuration is also found in some peptide-based antibiotics, including gramidicin S from *Bacillus* (Zimmer & Laland, 1975). Finally, in the anaerobe *Clostridium*, (*R*)-proline is apparently involved in the production of energy (Stadtman, 1954).

The biosynthesis of (*R*)-amino acids is usually accomplished by racemases or aminotransferases, to which pyridoxal phosphate (PLP)[2] is covalently attached via aldimine formation with an active-site lysine residue. For example, (*R*)-alanine, a major component of the peptidoglycan of both Gram-negative and Gram-positive bacteria, is obtained from (*S*)-alanine by enzymic racemization that is catalyzed by PLP-dependent alanine racemases (Walsh, 1989). The racemization is initiated by aldimine formation with the enzyme-bound PLP, and the α-hydrogen of the amino acid is subsequently abstracted, yielding a delocalized carbanion that reprotonates stereorandomly. Transimination with the active site lysine then releases the racemized alanine. In addition to the relatively well-studied alanine racemases, a PLP-dependent arginine racemase from *Pseudomonas* has been described, for which lysine and ornithine are also good substrates (Yorofuji et al., 1971). There are also reports of the existence in *Streptomyces* of two serine racemases, which also accept alanine as a substrate and evidently require PLP as a cofactor (Svensson & Gatenbeck, 1981).

A second group of enzymes that catalyze reactions of α-amino acids and their derivatives has been shown to contain an N-terminal pyruvoyl residue. All currently recognized pyruvoyl enzymes, which have been found in both bacteria and mammals, are either decarboxylases or reductases (van Poelje & Snell, 1990). The most thoroughly studied of these enzymes is the histidine decarboxylase from *Lactobacillus* 30a, which contains a pyruvoyl group that derives from an internal serine residue of the inactive proenzyme (Riley & Snell, 1970). The pyruvoyl cofactor performs mechanistically much like PLP in providing a delocalized electron sink for the substrate-derived carbanion. Indeed, histidine decarboxylase from the Gram-negative bacterium *Morganella*, as well as that from mammals, contains the more commonplace cofactor PLP (van Poelje & Snell, 1990). Although no pyruvoyl-dependent racemases have been discovered, their existence is

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[1] For all α-amino acids except cysteine, the classical L configuration is equivalent to (*S*).

[2] Abbreviations: PLP, pyridoxal phosphate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; ORF, open reading frame; LB, Luria broth; NAD^+/NADH, nicotinamide adenine dinucleotide/dihydronicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; Trien, triethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; INT, *p*-iodonitrotetrazolium violet; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; PCR, polymerase chain reaction; CD, circular dichroism; FPLC, fast protein liquid chromatography.

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plausible, given the apparent mechanistic equivalence of PLP and the pyruvoyl group as cofactors.

(*R*)-Phenylalanine occurs in nature as a component of gramicidin S, a cyclic decapeptide antibiotic that is produced by *Bacillus brevis*. Gramicidin S synthetase consists of two associated components, an ATP-dependent phenylalanine racemase and a second component that is responsible for the activation of the other amino acid constituents and for the subsequent peptide bond synthesis to form gramicidin S (Kanda et al., 1989). Phenylalanine racemase utilizes ATP in the formation of the aminoacyladenylate of (*S*)-phenylalanine. Subsequent nucleophilic attack by an active-site cysteine, with release of AMP, then forms an enzyme-bound thioester intermediate. The replacement of the electron-rich carboxylate with a thioester group facilitates the deprotonation at the 2-position that is required to bring about the change in configuration. Tyrocidine, another cyclic decapeptide antibiotic produced in some strains of *Bacillus*, is apparently biosynthesized in an analogous fashion. In fact, the protein sequences of the two synthetases show significant similarity (Hori et al., 1989), and their phenylalanine racemase components can be functionally exchanged in in vitro assays (Zimmer & Laland, 1975). Finally, penicillin biosynthesis in *Aspergillus nidulans* is initiated with δ-((*S*)-α-aminoadipyl)-(*R*)-cysteinyl–(*R*)-valine (ACV) synthetase. Initial studies indicate that this synthetase catalyzes the ATP-dependent formation of (*R*)-valine by epimerization of the (*S*)-valyl-enzyme thioester intermediate (van Liempt et al., 1989). It therefore seems that a common mechanism has evolved for the formation of the (*R*)-amino acids required in the synthesis of peptidyl antibiotics.

A final group of racemases is comprised of some rare enzymes that catalyze amino acid racemization in a cofactor-independent reaction. The best-known example, proline racemase from *Clostridium*, appears to require just two thiol groups, one from each of two identical subunits. Pioneering work from Abeles' group (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975) has suggested that one active-site thiol, acting as a base, abstracts the C-2 proton from the substrate and that the conjugate acid of the second active-site thiol delivers a solvent-derived proton to the opposite face.

The existence of a glutamate racemase activity in *Lactobacillus* was demonstrated as early as 1952 (Narrod & Wood, 1952; Ayengar & Roberts, 1952), and it was suggested that this enzyme does not require any cofactors (Ayengar & Roberts, 1952). We have long been intrigued by the mechanism and kinetics of cofactor-independent racemases, and we chose to investigate this glutamate racemase and to determine if it was truly devoid of any cofactor requirement. The enzyme has proved difficult to study, however, as it is relatively unstable and constitutes only a minute fraction of the total cellular protein in *Lactobacillus*. We decided, therefore, to clone the gene for glutamate racemase, with the aim of overexpressing the protein. Although the cloning of a glutamate racemase from a *Pediococcus* strain has been reported (Nakajima et al., 1986) and the enzyme is reported not to utilize PLP as a cofactor, no sequence data are available and mechanistic studies are nonexistent.

In this paper, we describe the purification to homogeneity of glutamate racemase from *Lactobacillus*, the cloning of the gene using standard hybridization techniques coupled with a novel selection for in vivo glutamate racemase activity, and the overproduction of the protein in *Escherichia coli*. We report the nucleotide sequence of the cloned gene and establish the cofactor independence of the overexpressed glutamate racemase. Sequence comparisons are used to implicate two cysteines as catalytically important residues.

EXPERIMENTAL PROCEDURES

Strains and Media. Lactobacillus fermenti (ATCC 9338) served as the source of both the purified glutamate racemase and the genomic DNA. *L. fermenti* cells were grown at 37 °C with shaking (200 rpm) in *Lactobacillus* MRS broth (Difco, Detroit, MI) after inoculation with starter cultures that had been grown overnight in the same nutrient broth at 37 °C. Cells from large-scale cultures were harvested by centrifugation after 16–20 h of growth. *E. coli* strain TG1 (Carter et al., 1985) was used as the host strain for the preparation of single-stranded DNA for sequencing. *E. coli* strain WM335 (Hoffmann et al., 1972) was the kind gift of Dr. Walter Messer. *E. coli* strain WM335 was grown in Luria broth (LB) medium supplemented with (*R*)-glutamic acid (100 μg/mL). *E. coli* strain DH5α MCR was utilized as the host strain for colony hybridization. *E. coli* strain DH5α was used as the host strain for the large-scale preparation of plasmid DNA (for sequencing and other manipulations) and for the large-scale purification of the overexpressed gene product. Transformants of *E. coli* were grown in LB media supplemented with ampicillin (200 μg/mL) and/or streptomycin (100 μg/mL).

Protein Determination. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as standard.

End-Point Assay for Glutamate Racemase Activity. During enzyme purification from *Lactobacillus*, an end-point assay for glutamate racemase activity was used. Samples were first incubated with saturating (*R*)-glutamate and then rapidly heated to inactivate the enzyme and assayed for (*S*)-glutamate using NAD^+/(*S*)-glutamate dehydrogenase. Typically, samples were incubated in 25 mM potassium phosphate buffer at pH 7.5, containing (*R*)-glutamic acid (38 mM), glycerol (10% v/v), bovine serum albumin (1 mg/mL), and dithiothreitol (2 mM) in a total volume of 100–200 μL for 30 min at 37 °C. Samples were then heated at 95–100 °C for 15 min. Denatured protein was removed by centrifugation in a microcentrifuge for 10 min at 14 000 rpm. The (*S*)-glutamate formed was quantitated by monitoring the change in A_{340nm} after the addition of (*S*)-glutamate dehydrogenase (30 units; units refer to the reverse reaction) and NAD^+ (10 mM) in 50 mM Tris-HCl, pH 9.5, at 30 °C. The extinction coefficient of NADH was taken as 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1968). Spectrophotometric measurements were made on a Hewlett Packard Model 4582A UV/vis spectrophotometer.

Enzyme Purification from Lactobacillus. Unless noted otherwise, all manipulations were performed at 4 °C. Triethanolamine (Trien)–2-(*N*-morpholino)ethanesulfonic acid (MES) buffers were prepared by titrating a prescribed concentration of Trien to the desired pH with MES. Stock solutions of pepstatin (5 mg/mL) in ethanol were used. Phenylmethanesulfonyl fluoride (100 mM) was prepared in ethanol immediately prior to use.

Pelleted cells (40 g) from a culture (4 L) of *L. fermenti* were suspended in 10 mM Tris-HCl, pH 8. The pH of the suspension was adjusted to neutrality by the addition of sodium hydroxide, and the cells were repelleted. After resuspension in the same buffer, the cells were pelleted again and were then frozen until needed. The frozen, pelleted cells were thawed and resuspended in 30 mM Trien–MES buffer, pH 7.5, (150 mL) containing (*R,S*)-glutamic acid (1 mM), glycerol (10% v/v), dithioerythritol (3 mM), pepstatin (1 mg/L), aprotinin

(1 mg/L), and phenylmethanesulfonyl fluoride (1 mM). Cells were lysed by two passes through a French pressure cell (Aminco, Silver Spring, MD) at 20 000 psi. Additional phenylmethanesulfonyl fluoride (1 mM) was combined with the cell lysate. Cell debris was removed by ultracentrifugation at 40 000 rpm for 2 h. The supernatant was diluted to 350 mL with (*R,S*)-glutamic acid (1 mM), pH 7.2, containing glycerol (10% v/v), dithioerythritol (3 mM), pepstatin (1 mg/L), and aprotinin (1 mg/L) and loaded onto a column (550 mL) of (diethylamino)ethylcellulose (DE-52, from Whatman, Hillsboro, OR) that had been preequilibrated with buffer A [50 mM Trien–MES buffer, pH 7.5, containing (*R,S*)-glutamic acid (1 mM), glycerol (10% v/v), dithioerythritol (3 mM), pepstatin (1 mg/L), and aprotinin (1 mg/L)]. The column was washed with buffer A (500 mL) and then eluted with a linear gradient (1000 mL + 1000 mL) of Trien–MES buffer (50 mM to 300 mM) containing the same additives as buffer A. Fractions containing glutamate racemase activity were pooled and concentrated by ultrafiltration through PM10 membranes (Amicon, Beverly, MA). The concentrated protein solution (2 × 25 mL) was frozen in liquid nitrogen and stored at −75 °C. Half of the protein solution (25 mL) was thawed, and the buffer was exchanged with buffer B [50 mM Trien–HCl buffer, pH 8.0, containing glycerol (10% v/v), (*R,S*)-glutamic acid (1 mM), and dithioerythritol (2 mM), by repeated concentration and dilution using a Centriprep-10 concentrator (Amicon).

Subsequent purification steps were performed using an FPLC system (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ). Portions of the protein were chromatographed on a Mono Q HR 10/10 FPLC anion-exchange column (10 × 100 mm, from Pharmacia-LKB Biotechnology Inc.) that had been preequilibrated with buffer B. After each application of protein the column was washed with buffer B (25 mL) and proteins were eluted with a linear gradient (37.5 mL + 37.5 mL) of NaCl (0–500 mM) in the same buffer at a flow rate of 2.5 mL/min. The fractions from the multiple runs containing glutamate racemase activity were pooled and concentrated by centrifugation using a Centriprep-10 concentrator. The concentrated protein solution was desalted by passage through a PD-10 column (Sephadex G-25M, from Pharmacia-LKB) that had been preequilibrated with buffer B. The protein solution was then applied to a Mono Q HR 5/5 FPLC anion-exchange column (5 × 50 mm, from Pharmacia-LKB). The column was washed with buffer B (5 mL) and was eluted with a linear gradient (7.5 mL + 7.5 mL) of NaCl (0–500 mM) in the same buffer at a flow rate of 0.5 mL/min. Eluted proteins, detected by A_{280nm}, were collected as fractions and assayed for racemase activity. A fraction, containing 6.4 units of racemase activity, was concentrated by centrifugation using a Centricon-10 microconcentrator, diluted to 1 mL with buffer C [10 mM potassium phosphate buffer, pH 7.6, containing (*R,S*)-glutamate (1 mM), glycerol (10% v/v), and dithiothreitol (0.44 mM)], and applied to a Toyo Soda Progel TSK HA-1000 hydroxylapatite column (7.5 × 75 mm, from Supelco, Bellafonte, PA) that had been equilibrated with buffer C. The column was washed with buffer C (6 mL) and then eluted with a linear gradient (15 mL + 15 mL) of potassium phosphate buffer (10 mM to 500 mM) containing the same additives as buffer C at a flow rate of 0.7 mL/min. The fractions containing glutamate racemase activity were combined, concentrated by centrifugation using a Centricon-10 microconcentrator, diluted to 1 mL with buffer D [50 mM Trien–HCl buffer, pH 7.0, containing (*R,S*)-glutamate (1 mM), glycerol (10% v/v), and dithiothreitol (0.3 mM)], and reconcentrated to about 100 μL. The protein solution was then diluted to 0.8 mL with buffer D and applied to a Mono Q HR 5/5 FPLC anion-exchange column that had been preequilibrated with buffer D. The column was washed with buffer D (10 mL), and the protein was eluted with a linear gradient (30 mL + 30 mL) of NaCl (0–300 mM) in the same buffer, at a flow rate of 1 mL/min. The fractions containing glutamate racemase activity were pooled and concentrated as above.

Purity Assessment and Characterization of the Racemase. The native molecular weight of glutamate racemase was deduced from the elution volume (compared with standards of known M_r) of racemase activity from a partially purified sample by size-exclusion chromatography using a Superose-12 HR 10/30 column (10 × 300 mm, from Pharmacia-LKB). The subunit molecular weight was determined by denaturing SDS–polyacrylamide gel electrophoresis. Isoelectric focusing gel (IEF) electrophoresis using Ampholine-PAG plates (from Pharmacia-LKB) was used to estimate the p*I* of the native racemase. After focusing of the pure protein on an IEF gel, slices (approximately 1 cm^2) of the unstained gel were incubated in assay solutions and the glutamate racemase activity was measured using the method described above. The purity of the protein was assessed by silver staining of both denaturing and native gels.

Amino Acid Composition and Sequencing. Total amino acid analysis by acidic hydrolysis/phenylisothiocyanate derivatization, digestion of the homogeneous racemase with trypsin, and amino acid sequencing by automated Edman degradation were performed by Dr. William S. Lane (Harvard University Microchemistry Facility, Cambridge, MA).

Plasmids and DNA Manipulations. The vector pUC18 (Yanisch-Perron et al., 1985) was used in library construction. The PCR-amplified region of *Lactobacillus* genomic DNA was subcloned into M13mp18 rf form DNA. Supercoiled plasmid DNA was prepared by the method of Del Sal et al. (1989). Routine methods of DNA manipulation were from Sambrook et al. (1989) or from Ausubel et al. (1987) unless otherwise noted.

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a MilliGen/Biosearch 7500 DNA synthesizer (MilliGen Corp., Bedford, MA) and purified using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). Concentrations were assessed from the absorbance at 260 nm. The following oligonucleotides (5′ to 3′) were synthesized:

08 GGGGGATCCIARRTCRAARTGRCC

09 GGGGAATTCTGGYTIGAACARCAYCARGC

012 CGACAACAACCACTTATTCACCCC-
GGCCCTTAAGTCAGGGAGTTGCCGT

where I indicates inosine, R indicates an equimolar mixture of deoxyadenosine and deoxyguanosine, and Y indicates an equimolar mixture of deoxycytosine and thymidine.

Isolation of Genomic DNA from Lactobacillus. Cells (1.8 g) were harvested from an 18 h culture (500 mL) of *L. fermenti*, washed with buffer E (0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA), repelleted by centrifugation from the same buffer, and frozen until needed. The frozen cells were thawed at 37 °C and resuspended in buffer E (19 mL). Genomic DNA from *Lactobacillus* was isolated by the procedure of Murray and Thompson (given in Ausubel et al. (1987)), incorporating the following change. During the lysis step, the cell suspension was incubated in the presence of

lysozyme (2 mg/mL) at 37 °C for 1 h, with occasional gentle mixing. The solution was frozen in liquid nitrogen and then thawed in warm water to increase the extent of cell lysis. The extract was then treated with proteinase K (0.6 mg/mL) for 30 min at 37 °C. The DNA was purified by ultracentrifugation in a cesium chloride density gradient and analyzed by electrophoresis through an agarose gel (0.6% w/v).

Use of PCR To Generate a Perfect Probe to the Racemase Gene. The amplification reactions [100 μL total volume overlaid with light mineral oil (50 μL)] were run in *Taq* DNA polymerase buffer (Promega, Madison, WI) containing equimolar amounts of the four dNTPs (0.2 mM each), priming oligonucleotides 08 and 09 (3 μM each), and *L. fermenti* genomic DNA (10 ng), using a programmable thermal cycler (PTC-100; MJ Research, Cambridge, MA). In the first cycle, samples were heated at 96 °C for 5 min and then taken to 37 °C for annealing. Following the addition of *Taq* polymerase (5 units) the temperature was increased (1 °C/5 s) to 72 °C and then held at 72 °C for 2 min. The subsequent 35 temperature cycles were 90 s at 94 °C, 2 min at 50 °C, and 2 min at 72 °C. After 35 cycles, the samples were heated for an additional 4 min at 72 °C. A control sample that lacked genomic DNA was treated in the same manner.

A portion (10 μL) of the above reaction mixture was analyzed by electrophoresis through an acrylamide gel (12% w/v). The remainder of the sample was diluted with water (0.3 mL), washed with an equal volume of Sevag's solution (Sambrook et al., 1989), and concentrated by centrifugation through an Ultra-Free MC 10K NMWL filter unit (Millipore, Bedford, MA). The concentrated sample was diluted with 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (0.4 mL) and then reconcentrated as before. The PCR product and M13mp18 plasmid DNA were each digested with both *Bam*HI and *Eco*RI. The PCR product was excised from an agarose gel [Sea Plaque (FMC Bioproducts, Rockland, ME) low-melting-temperature agarose (1%)/Nu Sieve GTG (FMC Bioproducts, Rockland, ME) agarose (3%)], and the digested dephosphorylated vector was excised from a low-melting-temperature agarose gel [Sea Plaque (1%)]. The isolated gel bands were copurified, coprecipitated, and ligated at 16 °C overnight with T4 DNA ligase. The ligation mixture was used directly to transform *E. coli* TG1 cells, and single-stranded DNA was prepared for sequencing.

DNA Sequencing. Sequencing of both single-stranded templates and of denatured double-stranded templates was performed by the Sanger method (Sanger et al., 1977) using the modified T7 DNA polymerase, Sequenase (U.S. Biochemical Corp., Cleveland, OH), according to the manufacturer's protocols. In some cases single-stranded binding protein (U.S. Biochemical Corp.) was used in sequencing reactions as directed by the manufacturer. The nucleotide sequences of regions containing high GC content were confirmed or ultimately resolved by using a thermal-cycling sequencing method with a modified *Taq* polymerase, Δ*Taq* (U.S. Biochemical Corp.), and 7-deaza-GTP according to the manufacturer's protocols.

Southern Blot. Genomic DNA from *Lactobacillus* (1 μg per sample per lane) that had been completely digested with a restriction endonuclease (*Ava*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, or *Sma*I) and electrophoresed through an agarose gel (0.7% w/v) was transferred to a Gene Screen nylon membrane (New England Nuclear Research Products, Boston, MA) using the capillary blot procedure described by the manufacturer. The oligonucleotide probe 012 was 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The blot was prehybridized at 50 °C for 24 h with 50 mM Tris-HCl buffer, pH 7.5, containing NaCl (1 M), sodium pyrophosphate (0.1% w/v), sodium dodecyl sulfate (1%, w/v), polyvinylpyrrolidone (molecular weight 40 000, 0.2% w/v), bovine serum albumin (0.2% w/v), ficoll (molecular weight 400 000, 0.2% w/v), dextran sulfate (molecular weight 500 000, 1% w/v), and denatured salmon sperm DNA (100 μg/mL). The ^{32}P-labeled oligonucleotide was purified using a column of Sephadex (NAP-5, from Pharmacia), and then used to probe the blot. The probe was hybridized with the blot for 24 h at 50 °C. The blot was washed according to the manufacturer's protocol and used for autoradiography.

Construction of Subgenomic Library. Genomic DNA (10 μg) from *Lactobacillus* was completely digested with *Bam*HI, and the resulting fragments of 1.5 kb to 2.5 kb were cut from an agarose gel and purifed using Gene Clean (BIO101, La Jolla, CA). The size-selected fragments were ligated into pUC18 that had been digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase.

Selection for Racemase Activity. Cells of *E. coli* strain WM335, which had been transformed with the subgenomic library by electroporation (850 V, 5 ms) using a BTX T-100 electroporation system (Biotechnologies and Experimental Research, Inc., San Diego, CA), were allowed to recover in LB media containing (*R*)-glutamic acid (100 μg/mL) for 1 h at 37 °C with gentle shaking. Cells were harvested by centrifugation for 60 s at 4000 rpm in a microcentrifuge. The pellet was resuspended in Luria broth (LB). A small portion was spread onto LB–agar plates (1.5% w/v) containing ampicillin (200 μg/mL), streptomycin (100 μg/mL), and (*R*)-glutamic acid (100 μg/mL). The remainder of the sample was spread onto selective agar plates that were identical to those described above but lacked (*R*)-glutamic acid.

Colony Hybridization. Transformants of *E. coli* strain WM335 that grew on the selective plates were transferred to nitrocellulose filters. The colonies on the replica filters were lysed, the filters were then neutralized, dried, baked, and washed, and the fixed DNA from the colonies was probed with the oligonucleotide 012 (that had been ^{32}P-labeled as described above) according to Woods (1984).

Purification of the Cloned Glutamate Racemase. The cells from an overnight culture (5 L) of *E. coli* DH5α (pKG3) were harvested by centrifugation. The pelleted cells (9.3 g) were suspended in buffer E [30 mM Trien–HCl buffer, pH 7.5, containing (*R,S*)-glutamic acid (1 mM), glycerol (10% v/v), dithiothreitol (2 mM), pepstatin (1 mg/L), aprotinin (1 mg/L), and phenylmethanesulfonyl fluoride (1.5 mM)]. Cells were lysed by two passes through a French pressure cell at 20 000 psi. Following ultracentrifugation at 40 000 rpm for 2 h to pellet the cell debris, the resulting supernatant was diluted with H_2O to a total volume of 88 mL and then loaded onto a column (80 mL) of (diethylamino)ethylcellulose (DE-52) that had been preequilibrated with buffer E. The column was washed with buffer E (300 mL) and then eluted with buffer E containing 200 mM NaCl (150 mL). The eluant was concentrated by ultrafiltration through a PM10 membrane. The concentrated protein solution was frozen in liquid nitrogen and stored at −75 °C. The protein solution was quickly thawed, and its buffer was exchanged with buffer B by repeated dilution and concentration using Centriprep-10 and Centricon-10 concentrators. Portions of this protein solution (approximately 30 mg of total protein in each portion) were applied to and eluted from a Mono Q HR10/10 FPLC anion-exchange column that had been preequilibrated with buffer B. After each application of protein, the column was

washed isocratically with buffer B (24 mL), and the column was developed with a linear gradient (19 mL + 19 mL) of NaCl (0–300 mM) in the same buffer, with a flow rate of 2 mL/min. The fractions containing racemase activity from the multiple runs were pooled and concentrated by ultrafiltration using a Centriprep-10 concentrator. The buffer of the concentrated protein solution was exchanged with buffer D as described above. The protein solution was then injected onto and eluted from a MonoQ HR10/10 FPLC ion-exchange column in multiple runs. After the protein was loaded, the column was washed with buffer D (24 mL) and then developed with a linear gradient (38 mL + 38 mL) of NaCl (0–150 mM) in buffer D. The fractions containing racemase activity were pooled and concentrated as described above.

Coupled Enzyme Assay for Glutamate Racemase Activity. The kinetic parameters of the overproduced protein were obtained at 30 °C with (*R*)-glutamate as the substrate using a continuous method based on a published aminotransferase assay (Rej, 1982). The racemase reaction is coupled to (*S*)-glutamate dehydrogenase/NAD^+ and diaphorase/*p*-iodonitrotetrazolium violet (INT). Rates were determined by following the absorbance at 500 nm in assay mixtures that contained 50 mM Trien–HCl buffer, pH 7.8, NAD^+ (5 mM), (*S*)-glutamate dehydrogenase (37.5 units), ADP (2.5 mM), INT (0.65 mM), diaphorase (2 units), (*R*)-glutamate, and glutamate racemase. Substrate concentrations ranged from 30 μM to 3 mM. The molar extinction coefficient of the reduced tetrazolium anion was determined by measuring the absorbance change at 500 nm induced by the oxidation of known amounts of NADH with diaphorase (2 units) in 50 mM Trien–HCl buffer, pH 7.8.

Circular Dichroism Assay for Glutamate Racemase Activity. The kinetic parameters for the cloned racemase were obtained at 30 °C with (*R*)-glutamate or (*S*)-glutamate as the substrate. Ellipticity was monitored using an Aviv 62DS CD spectrophotometer. The change in ellipticity at 204 nm with time was measured at substrate concentrations that ranged from 0.3 mM to 3 mM in assay mixtures that contained 10 mM potassium phosphate buffer, pH 8.0, dithiothreitol (0.2 mM), substrate, and glutamate racemase.

Spectrophotometric Analysis of Native Glutamate Racemase for the Presence of Pyridoxal Phosphate. Glutamate–oxaloacetate transaminase (also known as aspartate aminotransferase) (from Sigma, St. Louis, MO) (80 nmol, 3.7 mg) and glutamate racemase (35 nmol, 1 mg) were each repetitively exchanged into buffer E [50 mM potassium phosphate buffer, pH 8, containing dithiothreitol (0.5 mM)] by centrifugation through a Centricon-10 membrane, and finally were diluted with buffer E to a total volume of 0.5 mL to give final concentrations of 160 μM glutamate–oxaloacetate transaminase and 70 μM glutamate racemase. The absorbance spectrum from 240 nm to 500 nm of each sample of enzyme was measured. Two equivalents of pyridoxal phosphate were added to each sample, and the spectra were recorded again. The samples were kept at 4 °C overnight in the dark and then the buffer was exchanged four times as follows. Protein samples were diluted with buffer E (1.5 mL), allowed to sit at 4 °C in the dark for 45 min, and then concentrated as previously described. The racemase sample was then diluted to a concentration of 17.5 μM, and the aminotransferase to a concentration of 13.3 μM, and their spectra were measured as described earlier. The absorbance spectra were recorded using a Uvikon 860 UV/vis spectrophotometer (Kontron Instruments).

Hydrolysis Assay for the Presence of a Covalent Pyruvoyl Moiety. Parallel samples containing glutamate racemase (1 mg, 35 nmol), pyruvate (40 nmol), BSA (1 mg), or BSA (1 mg) plus pyruvate (40 nmol) were treated with 0.1 N NaOH at room temperature in a total volume of 400 μL for 2 h. Portions (180 μL) of each sample were diluted with potassium phosphate buffer, pH 7.5 (1 M, 90 μL), and neutralized with HCl (3 N, 10 μL). The pyruvate content of each sample was quantitated with lactate dehydrogenase (50 μg, about 50 units) by measurement of the oxidation of NADH (10 μM) at 340 nm in a total volume of 0.5 mL. The remainder of each sample was kept at room temperature for 72 h. Another portion (180 μL) from each sample was neutralized and assayed for the presence of pyruvate as described above.

Parallel samples identical to those above were incubated in 1.5 N HCl at 100 °C for 2 h. Portions (180 μL) of each sample were diluted with 1 M potassium phosphate buffer, pH 7.5 (160 μL) and neutralized with NaOH (6 N, 40 μL). The neutralized solutions were assayed for pyruvate as before. The remainder of each acidic sample was boiled for an additional 6 h. Another portion (180 μL) from each sample was then neutralized and assayed for the presence of pyruvate as detailed above.

Effect of Carbonyl Reagents on Glutamate Racemase Activity. To parallel samples of 100 mM Trien–HCl buffer, pH 8, containing glutamate racemase (about 1 μg, 0.15 unit) and dithiothreitol (0.25 mM), one of the following reagents was added to a final concentration of 10 mM: hydroxylamine, phenylhydrazine, sodium borohydride, or sodium cyanoborohydride. The stock solutions of hydroxylamine (100 mM) and phenylhydrazine (100 mM) were prepared in water and adjusted to pH 7.5. The stock solution of sodium borohydride (100 mM) was prepared in 1 mM NaOH. The stock solution of sodium cyanoborohydride (50 mM) was prepared in water. A second set of samples identical to those above but in which (*R*,*S*)-glutamate (3 mM) was included was also prepared. The samples containing phenylhydrazine or hydroxylamine, along with the appropriate control samples, were incubated at 30 °C for 30 min. The samples containing sodium cyanoborohydride, along with the appropriate control samples, were incubated at 37 °C for 30 min. Then samples containing sodium borohydride, along with the appropriate control samples, were incubated at room temperature for 30 min. Portions of each sample (20 μL) were assayed in a total volume of 1 mL using the coupled enzyme method described before.

Effect of EDTA on Glutamate Racemase Activity. Glutamate racemase activity was measured using the enzyme-coupled spectrophotometric assay. The assay mixture contained EDTA (10 mM) and subsaturating (*R*)-glutamate (0.3 mM). A control assay mixture contained no EDTA.

Database Searches and Protein Sequence Alignments. Searches of the GenBank and NBRF databases were performed using the GCG software package and the programs FASTA and TFASTA (Devereux et al., 1984). Detailed pairwise alignments were performed using the program GAP (Needleman & Wunsch, 1970).

RESULTS AND DISCUSSION

Purification and Preliminary Characterization of Glutamate Racemase from L. fermenti. To obtain sequence information for the synthesis of oligonucleotide probes to the glutamate racemase gene, the enzyme was purified to homogeneity. Glutamate racemase reportedly exhibits rather weak binding to its substrates (the published K_m values range from 2.2 mM (Diven, 1969) to 47 mM (Tanaka et al., 1961)), and no tight-

Table I: Purification of Glutamate Racemase from *L. fermenti*

	total catalytic act. (μmol min^{-1})	unit yield (%)	specific catalytic act. (μmol min^{-1} mg^{-1})	purifn factor	total protein (mg)
crude lysate	25.5	100	0.013	1	2000
DEAE-cellulose	14.5	57	0.10	8	145
Mono Q (pH 8.0)	6.4	25	5.8	450	1.1
hydroxylapatite	5.6	22	19	1460	0.3
Mono Q (pH 7.0)	4.1	16	158	12100	0.026

Table II: Sequences of Tryptic Peptides of Glutamate Racemase from *L. fermenti*[a]

01	W L L S G H F D L G T A Q I E E G D
02	S W L E Q H Q A T G N
03	K G P I G V I A M T A
04	M M V V A C N T A T A A A L P A L Q

[a] Peptides 02 and 03 were sequenced together from a mixture containing a 5:2 molar ratio (02:03).

binding enzyme inhibitors are known. Affinity purification approaches were therefore not attempted.

The enzyme is present at very low levels in *Lactobacillus* cell extracts as is evident in the purification scheme summarized in Table I. From 20 g of wet cell paste, after several chromatographic steps, approximately 25 μg of pure racemase was obtained in an overall unit yield of 16%, with an overall purification factor of over 12 000.

The final enzyme preparation was homogeneous both by isoelectric focusing (IEF) of the native protein and by denaturing gel electrophoresis. The native protein has a p*I* of about 6.5. On the basis of the elution volume of the racemase activity from a size-exclusion column (as compared with standards of known size), the molecular mass of the native racemase is estimated to be 30 000 Da. Silver-stained sodium dodecyl sulfate (SDS) gels of the pure protein show a single band of molecular mass about 30 000 Da. The active enzyme is therefore believed to be a monomer. A gel slice excised from an IEF gel, which corresponded to the single silver-stained band, contained racemase activity. No other portions of the gel showed any activity, suggesting that the pure protein is indeed glutamate racemase.

Preparation of a Subgenomic Library Containing the Full-Length Glutamate Racemase Gene from L. fermenti. To ensure the cloning of the full-length racemase gene as well as the expression of active enzyme, a sequence-derived hybridization screen and a functional selection scheme were employed in concert. The amino acid sequences of four peptides, obtained from proteolytic digests of the purified racemase, are shown in Table II. Since only a handful of protein-encoding genes from *Lactobacillus* have been cloned and sequenced (Schmidt et al., 1989; Pinter et al., 1988; Vanderslice et al., 1986; Porter & Chassy, 1988), no reliable codon usage frequencies have been established for this family of bacteria. A precise probe to the racemase gene was therefore first generated by using the polymerase chain reaction (PCR) (Mullis & Faloona, 1987) to amplify a region of the chromosomal racemase gene between guessed DNA sequences corresponding to portions of two tryptic peptide fragments of glutamate racemase. A pair of degenerate oligonucleotide primers (08 and 09, corresponding to regions of the tryptic peptides 01 and 02, respectively) with convenient restriction sites at the 5′-ends was designed to prime synthesis toward each other. The sequences of the oligonucleotide primers were based on the regions of the tryptic peptides that contain amino acids the codons for which have the least degeneracy. For positions that were ambiguous, an equimolar mixture of the two bases was incorporated. At positions where three or all four different bases were possible, inosine was incorporated. Using these primers, amplification by *Thermus aquaticus* (*Taq*) polymerase with total chromosomal DNA from *L. fermenti* as the template, generated a product of approximately 150 bp, which was cloned into an M13 vector. The sequence of the amplified DNA product agrees with the intervening amino acid sequences of the corresponding pair of tryptic peptides.

Several restriction digests of chromosomal DNA were then analyzed by Southern blotting (Southern, 1975), using a *precise* probe of 50 nucleotides (012) derived from the PCR amplification described above. This experiment showed that at least part of the racemase gene lies on a *Bam*HI fragment of approximately 2 kb, and a subgenomic library of 1.5–2.5 kb *Bam*HI fragments of *Lactobacillus* DNA was therefore constructed in the vector pUC18.

Selection for Glutamate Racemase Activity and Colony Hybridization. The mutant *E. coli* strain WM335 (Hoffmann et al., 1972) shows an absolute growth requirement for (*R*)-glutamic acid. Although the nature and the locus of the mutation are unknown, the use of the mutant host strain in principle provides a simple selection for glutamate racemase activity. *E. coli* strain WM335 grows on Luria broth (LB) supplemented with (*R*)-glutamic acid but does not grow on LB alone. Therefore, racemase-expressing clones are expected to grow on LB in the absence of (*R*)-glutamate, while cells lacking racemase activity should not be viable.

Electroporation was used to transform *E. coli* WM335 with the *Bam*HI subgenomic library described. From 13 000 transformants, three colonies grew on selective plates. These colonies were transferred to nitrocellulose membranes, and the labeled probe 012 was used to screen the plasmid DNA from these colonies to select for those containing the racemase gene.

Expression of Glutamate Racemase in the Selected Clones and Purification of the Overexpressed Cloned Enzyme. The growth characteristics and the levels of racemase activity of cells were examined upon transforming *E. coli* WM335 with the plasmids isolated from the selected clones. The most highly-expressing construct, designated pKG3, was used in all further work. Sequencing of the insert, combined with restriction mapping and anchored PCR analysis (Loh et al., 1989; Gulcher & Stefansson, 1988), revealed that pKG3 has a deletion of approximately 250 bp in the noncoding region downstream from the racemase gene and hence has lost one of the *Bam*HI sites at an insert–vector junction.

The purification scheme of glutamate racemase from *E. coli* DH5α (pKG3) is presented in Table III. On the basis of the specific catalytic activity of the purified enzyme compared with the activity in crude cell extracts, *E. coli* DH5α cells transformed with the plasmid pKG3 produce the racemase as 20–25% of the total soluble cell protein.

Table III: Purification of Glutamate Racemase from *E. coli* DH5α (pKG3)

	total catalytic act. (μmol min^{-1})	unit yield (%)	specific catalytic act. (μmol min^{-1} mg^{-1})	purifn factor	total protein (mg)
crude lysate	30 100	100	37.3	1	806
DEAE-cellulose	21 700	72	57.0	1.7	381
Mono Q (pH 8.0)	16 300	54	95.3	2.5	171
Mono Q (pH 7.0)	14 200	47	161	4.3	88

Full-Length Sequence of the Glutamate Racemase Gene. The nucleotide sequence of the racemase gene and the upstream noncoding region show two possible in-frame translational start sites (ATG codons) separated by 24 nucleotides. The open reading frames that follow encode proteins of 268 and 260 amino acids, respectively. It is not obvious, a priori, which of these two methionine residues constitutes the natural start codon in *Lactobacillus*. The racemase gene (which was cloned into a pUC vector) is expressed at high levels in *E. coli*. It is thus reasonable to assume that a *Lactobacillus* promoter precedes the racemase gene in the expressing construct and that this promoter functions in the *E. coli* host. The form of the racemase that is produced in *E. coli* is thus likely to be the same as that produced in *Lactobacillus*. To discover the start codon of the glutamate racemase gene, the sequence of the first five N-terminal amino acids of the homogeneous cloned glutamate racemase was determined and found to be Met-Asp-Asn-Arg-Pro-. The first 5′-methionine of the open reading frame evidently serves as the start codon of glutamate racemase.

The nucleotide sequences of the racemase gene and of its derived amino acid sequence are shown in Figure 1. The open reading frame encodes 268 amino acids to yield a protein of molecular mass 28 300 Da. The locations of the four tryptic peptides, as well as of the N-terminal peptide, are underlined. The sequences of the four tryptic peptides are fully confirmed by the gene sequence.[3]

Continuous Spectrophotometric Assay for Glutamate Racemase Activity. To determine the kinetic constants for glutamate racemase, it was first necessary to develop a continuous assay for racemase activity. The use of (*S*)-glutamate dehydrogenase as a coupling enzyme is hampered by an equilibrium constant for the coupling system that disfavors amino acid oxidation. The use of high concentrations of NAD^+ and of high pH in the assay mixture helps to drive the reaction toward the oxidation of (*S*)-glutamate, but even at pH 8.5 effective coupling cannot be achieved. A continuous assay for the racemase reaction at pH 7.8 was developed, however, using a glutamate dehydrogenase/diaphorase-coupled system. In this assay, the glutamate dehydrogenase reaction is rendered irreversible by the continuous removal of NADH, the oxidation of which is catalyzed by diaphorase using *p*-iodonitrotetrazolium violet (INT) as a cosubstrate. This system is similar to an assay for glutamate aminotransferase (Rej, 1982). Under the conditions used for the racemase assay, the molar extinction coefficient of the reduced tetrazolium anion at 500 nm was determined to be 14 300 M^{-1} cm^{-1}. The use of this assay suffers two drawbacks. First, only (*R*)-glutamate can serve as the substrate. Second, the thiols that are required to stabilize glutamate racemase (Tanaka et al. 1961; Diven, 1969) reduce the INT directly. However, by diluting the enzyme immediately prior to use into buffer containing a low concentration of dithiothreitol, and by further dilution into an assay mixture that contains no added thiols, this problem can be circumvented with essentially no loss of racemase activity.

Continuous Circular Dichroism Assay for Glutamate Racemase Activity. In the coupled assay described above, (*R*)-glutamate serves as the substrate. No such coupled assay using commercially available enzymes with (*S*)-glutamate as the substrate appeared to be feasible. In an effort to use polarimetry (such as was employed by Cardinale and Abeles (1968) for proline racemase), the optical rotation of a neutral solution of 100 mM glutamic acid was measured and found to be only 0.11° at 365 nm. This is some 30-fold lower than that of the same concentration of proline at the same pH. This striking difference has previously been noted (Hayashi et al., 1966), and it effectively rules out a simple polarimetric assay for glutamate racemase.

All aliphatic (*S*)-amino acids display a strong positive Cotton effect in the far ultraviolet. This absorption, which is centered near 203 nm when the amino acid is in its zwitterionic form, has generally been attributed to an n-to-π* transition of the α-carboxylate group (Toome & Weigele, 1981). While the far ultraviolet is generally inaccessible with ordinary polarimeters, modern CD spectrometers can be used to monitor the circular dichroism at wavelengths as low as 190 nm. The molar ellipticity of glutamic acid at 204 nm and pH 8.0 was determined to be 31.0 mdeg cm^{-1} mM^{-1}, in reasonable agreement with the reported value at 203 nm and pH 7 of 33.7 mdeg cm^{-1} mM^{-1} (Katzin & Gulyas, 1968). Using a CD assay, therefore, the activity of glutamate racemase can be continuously monitored using either enantiomer of glutamic acid as substrate.

Determination of the Kinetic Constants for Glutamate Racemase. The steady-state kinetic parameters, k_{cat} and K_m, for glutamate racemase with (*R*)-glutamate as the substrate were determined using both the coupled enzyme assay and the CD method. The CD assay alone was utilized to measure these parameters with (*S*)-glutamate as the substrate. Using the coupled enzyme assay at 30 °C, the K_m for (*R*)-glutamate was determined to be 0.24 mM. A single point measurement at 30 °C performed under saturating conditions (about 25 times K_m) gave a k_{cat} of 76 s^{-1}. These numbers are in reasonable agreement with those obtained from the CD assay, which were 0.26 mM and 68 s^{-1}, respectively. With (*S*)-glutamate as the substrate, a K_m of 0.33 mM and a k_{cat} of 69 s^{-1} was measured. Error limits of ±30% for K_m and ±15% for k_{cat} are estimated. The kinetic constants for (*R*)-glutamate as the substrate are therefore indistinguishable from those of (*S*)-glutamate. The parameters from the CD assay yield k_{cat}/K_m values of (2.2–2.5) × 10^5 M^{-1} s^{-1}.

The kinetic constants of glutamate racemase from *L. fermenti* reported here differ substantially from those published for the cloned glutamate racemase from *Pediococcus* (Nakajima et al., 1988). Nakajima et al. (1988) report K_m values for (*R*)- and (*S*)-glutamate of 14 and 10 mM, respectively, at pH 7.4. The k_{cat} values obtained in the present work at 30 °C are 2–3-fold higher than the corresponding values determined at 37 °C by Nakajima et al. (1988). The values of

[3] On the basis of the gene sequence, a single amino acid position in each of the two tryptic peptides that copurified and were consequenced had been misassigned.

```
1
ATG GAC AAT CGC CCA ATT GGA GTG ATG GAT TCT GGC TTG GGG GGC CTT TCA GTC GTC CGG
met asp asn arg pro ile gly val met asp ser gly leu gly gly leu ser val val arg
61
GTT ATC CAG CAA AAA CTA CCC AAC GAA GAG GTC ATC TTC GTG GGC GAC CAA GGT CAC TTC
val ile gln gln lys leu pro asn glu glu val ile phe val gly asp gln gly his phe
121
CCG TAC GGA ACC AAG GAT CAA GCG GAG GTC CGT CAA CTG GCG CTT TCG ATT GGT GCT TTT
pro tyr gly thr lys asp gln ala glu val arg gln leu ala leu ser ile gly ala phe
181
TTA CTC AAG CAT GAC GTA AAG ATG ATG GTC GTG GCC TGC AAT ACG GCG ACG GCG GCC GCA
leu leu lys his asp val lys met met val val ala cys asn thr ala thr ala ala ala
241
CTG CCG GCC CTG CAA GCG GCG CTC CCA ATT CCC GTA ATT GGG GTC ATT GAG CCC GGC GCC
leu pro ala leu gln ala ala leu pro ile pro val ile gly val ile glu pro gly ala
301
CGG GCG GCC CTG GCC CAG GAT AAA AAG GGA CCG ATT GGG GTG ATC GCC ACC ACG GCA ACC
arg ala ala leu ala gln asp lys lys gly pro ile gly val ile ala thr thr ala thr
361
ACC ACG GCC GGC GCC TAC CCA GCT ACG ATC GAA CGG CTA GCG CCG GGT ACC CCG GTC ATT
thr thr ala gly ala tyr pro ala thr ile glu arg leu ala pro gly thr pro val ile
421
GCA AAG GCC ACC CAG CCA ATG GTC GAA ATC GTT GAG CAC GGC CAG ACC GGG ACG GCG AAG
ala lys ala thr gln pro met val glu ile val glu his gly gln thr gly thr ala lys
481
GCC CAA GAA GTG GTT AGT GAA CAG CTA ATG ACC TTT AAA GAG CAC CCG GTA AAA ACC CTG
ala gln glu val val ser glu gln leu met thr phe lys glu his pro val lys thr leu
541
ATT ATG GGC TGC ACC CAC TTC CCG TTT TTG GCC CCG GAA ATA AGT AAG GCG GTC GGA CCA
ile met gly cys thr his phe pro phe leu ala pro glu ile ser lys ala val gly pro
601
ACG GTG GCC TTA GTC GAC CCG GCT AAG GAA ACG GTG GCC ACG GCT AAG TCC TGG CTG GAA
thr val ala leu val asp pro ala lys glu thr val ala thr ala lys ser trp leu glu
661
CAG CAC CAA GCA ATG GGT AAC CAC GCC CAC CCC AAC TAC CAC CTA TAT TCA ACG GGC AAC
gln his gln ala met gly asn his ala his pro asn tyr his leu tyr ser thr gly asn
721
CTC CCT GAC TTA AGG GCC GGG GTG AAT AAG TGG TTG TTG TCG GGG CAC TTT GAC TTA GGA
leu pro asp leu arg ala gly val asn lys trp leu leu ser gly his phe asp leu gly
781
ACC GCC CAA ATT GAA GAG GGG GAT TAA
thr ala gln ile glu glu gly asp OCH
```

FIGURE 1: Nucleotide sequence of the gene that encodes glutamate racemase (GenBank accession no. LO2916). The nucleotide numbering begins at the initiation codon of the reading frame. The translated amino acid sequence is shown beneath the corresponding codons. The regions of the amino acid sequence that are underlined were also determined by Edman degradation of the purified protein.

Table IV: Pyruvate Content of Glutamate Racemase

	% theoretical amount of pyruvate after	
	acidic hydrolysis	alkaline hydrolysis
pyruvate	97.3	97.2
pyruvate + BSA	100.5	103.6
glutamate racemase	4.5	0

k_{cat}/K_m for the *Lactobacillus* enzyme are thus about 100-fold higher than those reported for the enzyme from *Pediococcus*.

Investigation of Possible Cofactor Requirements. The early studies on glutamate racemase by Ayengar and Roberts (1952) suggested that the enzyme from *Lactobacillus* does not require pyridoxal phosphate, but the presence of a tightly bound cofactor could not be ruled out. Other workers reported that hydroxylamine weakly inhibits the racemase, either competitively (Glaser, 1960) or in a pH-dependent noncompetitive fashion (Diven, 1969), and speculation continued about the involvement of pyridoxal phosphate as a cofactor. In other studies of glutamate racemase, hydroxylamine was found not to inhibit glutamate racemase in assays at pH 7.5 (Tanaka et al., 1961). Indeed, on the basis of the absorption and fluorescence spectra of what may have been an impure enzyme preparation, glutamate racemase was claimed to be a flavin-containing protein (Tanaka et al., 1961). Later studies (Diven, 1969) indicated not only that flavin was not a cofactor for glutamate racemase but also that flavin and several flavin analogues are weak inhibitors of the enzyme. A more recent report shows that the glutamate racemase from *Pediococcus*

```
                                                MDNRPIGVMDSGLGGLSVVRVIQQKLPN
                                                ....: |:|||:|||||   |.: ||:
MRQSMATKLQDGNTPCLAATPSEPRPTVLVFDSGVGGLSVYDEIRHLLPD

EEVIFVGDQGHFPYGTKDQA.EVRQLALSIGAFLLKHDVKMMVVACNTAT
 . |:. |.. ||||.|.:|  | .:.  :.|.  :..: : |||||||.
LHYIYAFDNVAFPYGEKSEAFIVERVVAIVTAVQERYPLALAVVACNTAS

AAALPALQAALPIPVIGVIEPGARAALAQDKKGPIGVIATTATTTAGAYP
...||||.. :.:||:||: |: ::|   . .| :|:::|| :|.. :  .
TVSLPALREKFDFPVVGVV.PAIKPAARLTANGIVGLLATRGTVKRSYTH

ATIERLAPGTPVIAKATQPMVEIVE...HGQTGTAKAQEVVSEQLMTFKE
. |.|:| :..:   :...|||:.|   ||:... .| . :  .:: :||
ELIARFANECQIEMLGSAEMVELAEAKLHGEDVSLDALKRILRPWLRMKE

HPVKTLIMGCTHFPFLAPEISKAVGPTVALVDPAKETVATAKSWLEQH..
.| .|:::|||||:|..|: ..:.... |||.: ...  . .:|||:.
PP.DTVVLGCTHFPLLQEELLQVLPEGTRLVDSGAAIARRTAWLLEHEAP

QAMGNHAHPNYHLYSTGNLPDLRAGVNKWLLSGHFDLGTAQIEEGD
:|.:..|: .: :  |.. .:| :.:.:: :..  .|:. .
DAKSADANIAFCMAMTPGAEQLLPVLQRYGFETLEKLAVLG
```

FIGURE 2: Alignment of the amino acid sequence of glutamate racemase (upper row) with the translated sequence of ORF1 (lower row).

```
Asp Racemase    ..F I V L T C N T A H...K V I L G C T E L S L M N..
                  .     .   . .       .     .   .
Glu Racemase    ..M M V V A C N T A T...T L I M G C T H F P F L A..
                  . . . . . . .       .     . . . . . . .
ORF1            ..L A V V A C N T A S...T V V L G C T H F P L L Q..
```

FIGURE 3: Comparison of the cysteine-containing regions of the deduced amino acid sequences of aspartate racemase from *Streptococcus thermophilus*, glutamate racemase from *Lactobacillus fermenti*, and ORF1 from the *rrnB* ribosomal RNA operon of *Escherichia coli*.

does not contain pyridoxal phosphate (Nakajima et al., 1988), but the possibility of a pyruvoyl cofactor was not considered. Since *Lactobacillus* was the source of the enzyme in all of the early studies, it was important to reinvestigate the cofactor requirements of this enzyme.

All known pyridoxal phosphate-containing enzymes bind the cofactor via an imine linkage to the ϵ-amino group of a lysine at the active site (Walsh, 1979). This chromophore has an absorption maximum between 330 and 420 nm with an extinction coefficient ranging from 6000 to 10 000 M^{-1} cm^{-1} depending on the enzyme and the protonation state of its coenzyme aldimine (Morino & Nagashima, 1984). The absorption spectrum of glutamate racemase was therefore compared with that of the well-characterized pyridoxal phosphate-dependent enzyme, aspartate aminotransferase from porcine liver. The spectrum of the aminotransferase shows the expected absorbance maximum at 366 nm (pH 8.0), whereas the spectrum of an equimolar solution of glutamate racemase lacks any significant absorbance at wavelengths higher than 310 nm. Attempts to "reconstitute" the racemase with exogenous pyridoxal phosphate failed, and added cofactor had no effect on the reaction rate. These results confirm that glutamate racemase from *L. fermenti* does not contain pyridoxal phosphate or require this cofactor for activity.

The presence of a covalently-bound pyruvoyl cofactor has been established in several decarboxylases and reductases (van Poelje & Snell, 1990). No pyruvoyl-dependent racemases have been found, yet their existence seems possible given the apparent mechanistic equivalence of the pyruvoyl group and PLP. The pyruvoyl moiety may be attached via an amide (Riley & Snell, 1968) or an ester linkage (Yang & Abeles, 1987; Scandurra et al., 1987) and may be liberated with either acid or base hydrolysis to yield free pyruvate.

To determine whether glutamate racemase contains a covalently-linked pyruvoyl group, the purified racemase was treated either with acid or with base, according to the earlier work, and then assayed for pyruvate with lactate dehydrogenase/NADH. Control incubations containing pyruvate and bovine serum albumin, and pyruvate alone, were subjected to hydrolysis conditions identical to those used for the racemase. The results of these experiments are summarized in Table IV. No pyruvate was detected in either of the racemase-containing solutions. More extended hydrolytic incubations also failed to yield any detectable pyruvate, indicating that neither an amide-linked nor an ester-linked pyruvoyl moiety is present in the enzyme.

Many pyruvoyl-containing and pyridoxal phosphate-dependent enzymes are sensitive to sodium borohydride, hydroxylamine, or phenylhydrazine. Since these reagents can react with imines as well as with ketones, they have been used to inactivate pyruvoyl-enzymes as well as PLP-containing enzymes, in the presence or absence of substrates (van Poelje & Snell, 1990). Solutions containing glutamate racemase were incubated with each of these reagents, both in the presence and in the absence of saturating substrate. Only incubation with phenylhydrazine significantly affected the activity of glutamate racemase. Yet, pyruvoyl-phenylhydrazones have a characteristic absorbance spectrum (Riley & Snell, 1968) that is completely lacking in phenylhydrazine-treated glutamate racemase, thus ruling out the possibility that the mode of inactivation of glutamate racemase by phenylhydrazine involves the formation of a pyruvoyl-phenylhydrazone.

Glutamate racemase does not require the addition of metal ions for activity, and the enzyme activity is unaffected by the inclusion of 10 mM EDTA in assay mixtures. This suggests that the reaction of glutamate racemase does not involve a metal ion, though the possibility of the involvement of a tightly bound metal ion cannot be eliminated.

Stabilization of Glutamate Racemase. Glutamate racemase is stabilized by the presence of reduced thiols. After 3 h at 30 °C, samples of enzyme containing either dithiothreitol or glutamate or both showed about 80% retention of the original activity, whereas samples that lacked both dithiothreitol and glutamate retained less than 40% of the starting activity. The thiol alkylating agent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), rapidly and completely inactivates glutamate racemase, as has been shown by others (Tanaka et al., 1961; Nakajima et al., 1988). Since the presence of substrate or dithiothreitol alone protects the enzyme from inactivation to nearly the same extent as the two in combination, it is reasonable to suggest that the reduced thiol(s) that is(are) critical for enzyme activity lies(lie) near the substrate binding site and may play a catalytic role in the racemase reaction. Furthermore, in each of the cofactor-independent amino acid "racemases", proline racemase (Cardinale & Abeles, 1968), hydroxyproline racemase (Finlay & Adams, 1970), and diaminopimelic acid epimerase (Wiseman & Nichols, 1984), cysteine residues have been implicated as the likely catalytic bases. Since the amino acid sequence of glutamate racemase reveals the presence of two cysteine residues, it seems plausible that one or both of these cysteines acts as the base(s) in 2-proton abstraction from the substrate.

Database Searches for Amino Acid Sequence Similarity. A search of the NBRF protein data bank failed to identify any protein sequences that are similar to the sequence of glutamate racemase. The program TFASTA was then used to translate all six frames of the *nucleotide* sequences contained in the GenBank database and to find hypothetical translated sequences that contain regions bearing similarities to the amino acid sequence of glutamate racemase. Interestingly, an unidentified open reading frame (ORF) in the *rrnB* ribosomal RNA operon of *E. coli* was found to be 30% identical to that of glutamate racemase. The pairwise alignment of the two sequences obtained using the program GAP is shown in Figure 2. This ORF, designated ORF1 (Brosius et al., 1981), encodes a putative protein of 289 amino acids. The *rrnB* operon also encodes the genes for 16S, 23S, and 5S rRNA, a second unidentified ORF, and the gene for glutamyl-$tRNA_2$. It is tempting to speculate that the ORF1 codes for glutamate racemase, even though glutamate racemase has never been identified in *E. coli*. Alternatively, the ORF1 gene may code for another cofactor-independent racemase.

The argument for the catalytic importance of the two cysteine residues becomes more compelling in light of the recently published sequence of aspartate racemase from

Streptococcos thermophilus (Yohda et al., 1991), this enzyme appearing to be a new member of the growing family of cofactor-independent racemases (Okada et al., 1991). Using the sequence alignment program GAP, the sequence of this protein is 24% identical to the sequence of glutamate racemase from *Lactobacillus*. The two cysteine residues in glutamate racemase and their surrounding regions are well-conserved both in the aspartate racemase sequence and in the deduced sequence of ORF1, as shown in Figure 3. The amino acid sequences of ORF1 and aspartate racemase are 22% identical.

ADDED IN PROOF

The *E. coli* open reading frame ORF1 (Brosius et al., 1981) that is so similar in sequence to the glutamate racemase from *Lactobacillus* reported here has recently been identified as *murI*, the inactivation of which causes a requirement for exogenous (*R*)-glutamate (Doublet et al. 1992). Further, one of the lesions in the strain we have used for selection, *E. coli* WM335, has just been mapped (Dougherty et al., 1993) to the same locus (i.e., that of ORF1). It appears, therefore, that cofactor-independent glutamate racemase occurs in *E. coli* and is a more ubiquitous enzyme than has been commonly believed.

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