Evolution of Enzymatic Activities in the Enolase Superfamily: Functional Assignment of Unknown Proteins in *Bacillus subtilis* and *Escherichia coli* as L-Ala-D/L-Glu Epimerases†

Dawn M. Z. Schmidt, Brian K. Hubbard, and John A. Gerlt*

Departments of Biochemistry and Chemistry, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: The members of the mechanistically diverse enolase superfamily catalyze different overall reactions by using a common catalytic strategy and structural scaffold. In the muconate lactonizing enzyme (MLE) subgroup of the superfamily, abstraction of a proton adjacent to a carboxylate group initiates reactions, including cycloisomerization (MLE), dehydration [*o*-succinylbenzoate synthase (OSBS)], and 1,1-proton transfer (catalyzed by an OSBS that also catalyzes a promiscuous *N*-acylamino acid racemase reaction). The realization that a member of the MLE subgroup could catalyze a 1,1-proton transfer reaction, albeit poorly, led to a search for other enzymes which might catalyze a 1,1-proton transfer as their physiological reaction. YcjG from *Escherichia coli* and YkfB from *Bacillus subtilis*, proteins of previously unknown function, were discovered to be L-Ala-D/L-Glu epimerases, although they also catalyze the epimerization of other dipeptides. The values of k_{cat}/K_M for L-Ala-D/L-Glu for both proteins are $\sim 10^4$ M^{-1} s^{-1}. The genomic context and the substrate specificity of both YcjG and YkfB suggest roles in the metabolism of the murein peptide, of which L-Ala-D-Glu is a component. Homologues possessing L-Ala-D/L-Glu epimerase activity have been identified in at least two other organisms.

The growing sequence and structural databases provide the information for understanding the interplay among sequence, structure, and function in the evolution of new enzymes. We have focused on understanding the evolution of activities in the enolase superfamily, an example of a "mechanistically diverse" superfamily whose members catalyze different reactions that share a common partial reaction, i.e., generation and stabilization of an enolate anion intermediate (*1*, *2*). Each member of the superfamily characterized to date uses a conserved general basic catalyst to abstract a proton from the carbon adjacent to a carboxylic acid (α-proton). At least one carboxylate oxygen of the substrate is directly coordinated to a conserved and catalytically essential divalent metal ion (usually Mg^{2+}). Accordingly, formation of the enolate anion intermediate is accompanied by an enhanced electrostatic interaction between the enolate anion and cationic metal ion, thereby providing differential stabilization of the intermediate and allowing it to be kinetically competent.

The members of the enolase superfamily share a common fold: a "capping domain" formed by the N- and C-terminal segments of the polypeptide and an "internal" $(\beta/\alpha)_7\beta$-barrel domain (*3*, *4*). Most of the functional groups that determine substrate specificity are located in the capping domain; the general acid/base catalysts that mediate proton transfer reactions and the ligands for the essential divalent metal ion are located in the barrel domain. This structure provides an ideal scaffold for evolution of new function, because substrate recognition and reaction chemistry are located in separate structural units where these functions can evolve independently. Furthermore, the metal ion ligands and acid/base catalysts are located individually at the ends of the β-strands within the $(\beta/\alpha)_7\beta$-barrel domain so their identities and spatial relationships also can be varied independently.

Enolase, mandelate racemase (MR), and muconate lactonizing enzyme (MLE) have served as the prototypic members of the enolase superfamily; the homologues in the protein databases can be assigned to distinct subgroups on the basis of the identities of (1) the ligands for the essential divalent metal ion and (2) the general basic catalysts that initiate the reaction by abstraction of the α-proton of the substrate (*5*). With a focus on the MLE subgroup, each member has a Lys-X-Lys motif at the end of the second β-strand and a Lys at the end of the sixth β-strand. In the case of MLE, the second Lys of the Lys 167-X-Lys 169 motif is thought to be the general acid catalyst that protonates the α-carbon of the muconolactone product (abstracts the α-proton in the reverse reaction that generates *cis*,*cis*-muconate); Lys 273 at the end of the sixth β-strand is thought to be a "spectator" in catalysis, although substitutions at this position compromise the rate of the reaction. In the case of *o*-succinylbenzoate synthase (OSBS)[1] from *Escherichia coli* that catalyzes a dehydration reaction, the available structural

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* To whom correspondence should be addressed: Department of Biochemistry, University of Illinois, 600 S. Mathews Ave., Urbana, IL 61801. Phone: (217) 244-7414. Fax: (217) 265-0385. E-mail: j-gerlt@uiuc.edu.

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information suggests that Lys 235 at the end of the sixth β-strand is the general base that abstracts the α-proton and the second Lys of the Lys 131-X-Lys 133 motif is thought to be the general acid that facilitates departure of the hydroxide leaving group (*3*); mechanistic and additional structural studies to examine these hypotheses are in progress.

A protein from a species of *Amycolaptosis* discovered in a search for an "*N*-acylamino acid racemase" (NAAAR) is a member of the MLE subgroup of the enolase superfamily (*6*). That protein is an inefficient catalyst of the racemization reaction ($k_{cat}/K_m = 3.7 \times 10^2\ M^{-1}\ s^{-1}$). Using clues derived from the genomic context of a homologue encoded by the *Bacillus subtilis* genome, we demonstrated that the "NAAAR" is much more efficient in catalyzing the OSBS reaction ($k_{cat}/K_m = 2.5 \times 10^5\ M^{-1}\ s^{-1}$), with the likely physiological role of the protein being an OSBS rather than an NAAAR (*7*). We rationalized the ability of the same protein to catalyze two different reactions with the expectation that each requires the participation of two general acid/base catalysts on opposite faces of the active site, e.g., the Lys residues at the ends of the second and sixth β-strands, in the formation of an enolate anion intermediate and its conversion to product.

This discovery of a "promiscuous" enzyme that catalyzes two different reactions lends considerable support to the hypothesis that "new" enzymes evolve by selection of a progenitor that adventitiously catalyzes the desired new reaction, albeit at a very low rate but a rate sufficient to provide a selective advantage (*8*). Subsequent evolution enhances the "new" reaction, likely at the expense of the "old" reaction. The promiscuous reaction likely is silent in the normal cellular context, but the gene encoding the promiscuous enzyme is available for duplication and evolution to enhance the "accidental" reaction so that it can be used in a new metabolic context.

The *E. coli* and *B. subtilis* genomes each encode members of the MLE subgroup that were annotated as functionally unknown: YcjG in *E. coli* and YkfB in *B. subtilis*. Realizing that members of the MLE subgroup are capable of catalyzing a 1,1-proton transfer reaction, the accidental NAAAR reaction, we hypothesized that one or both of these might catalyze 1,1-proton transfer reactions, albeit on unknown substrates. Using clues derived from the genomic context of the encoding genes, we have determined that both YcjG and YkfB are L-Ala-D/L-Glu epimerases but do not catalyze either the NAAAR or OSBS reaction.

MATERIALS AND METHODS

Restriction enzymes were purchased from Gibco BRL or New England Biolabs. T4 DNA ligase and T4 DNA kinase were purchased from Gibco BRL. Oligonucleotide primers were synthesized by Bio-Synthesis (Lewisville, TX), and DNA sequencing was performed by the University of Illinois Biotechnology Center. Dipeptides were purchased from Sigma or Bachem. Dipeptides not commercially available were synthesized as described below. Alanine dehydrogenase and diaphorase were purchased from Sigma/Aldrich, as were all other reagents of the highest quality grade commercially available.

^{1}H NMR spectra were recorded with a Varian Unity Inova NB 500 MHz NMR spectrometer. A Bio-Pilot or AKTA FPLC system (Pharmacia Biotech) was used for purification of proteins. Resins for column chromatography were purchased from Pharmacia Biotech. Purified proteins were concentrated using an Amicon stirred ultrafiltration cell with 10000 NMWL Biomax High Flow PES ultrafiltration membranes (Millipore). Protein concentrations were determined using extinction coefficients given by the ProtParam tool (www.expasy.ch). Spectrophotometric assays were performed with a Perkin-Elmer Lambda 2S spectrometer. A Beckman HPLC system was used. Electrospray ionization (ESI) mass spectrometry was performed on a Quattro mass spectrometer at the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory. Kinetic constants were determined using the CLELAND suite of programs (*9*).

Cloning, Expression, and Purification of YcjG. Oligonucleotide primers containing the first and last 24 bases of each gene as well as the 5′ recognition sequence for *Nde*I and the 3′ recognition sequence for *Bam*HI were used to PCR amplify the *ycjG* gene from *E. coli* strain MG1655 genomic DNA using Pfu DNA polymerase (Stratagene). The amplified product was isolated using the Qiagen gel extraction kit (Qiagen, Inc.), digested with *Nde*I and *Bam*HI, and ligated into the same sites of a modified version of the pET15B vector. This expression vector has been modified to express a 10-histidine tag rather than the usual six-histidine tag. The sequence of the gene was verified by dideoxynucleotide sequencing. The His-tagged protein was expressed in *E. coli* strain BL21(DE3).

The purification of YcjG will serve as a model for the purification of other His-tagged proteins in this work. Transformed cells were grown at 37 °C in 2 L of LB to OD_{600} of 0.6 and induced with 0.5 mM IPTG. The cells were allowed to grow for an additional 24 h before harvesting by centrifugation (5000 rpm at 4 °C) in a Sorvall JA-10 rotor. The cells were lysed by sonication (Fisher Scientific 550 Sonic Dismembrator). The cell lysate was applied to a Chelating Sepharose Fast Flow column (16 mm × 24 cm) in binding buffer [5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)]. The column was washed with binding buffer and wash buffer [60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)], and YcjG was eluted with 400 mL of a 1:1 mixture of binding buffer and strip buffer [100 mM EDTA, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)]. The purified protein was concentrated and dialyzed into 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5). Long-term storage of YcjG was in 40% glycerol at −20 °C. The mass of the protein was confirmed by ESI-MS (expected mass of 36 906.3, observed mass of 36 908.8 ± 36.9).

To remove the 10-histidine tag from YcjG for crystallographic studies, the protein was dialyzed against 1× PBS (*10*). Thrombin (Pharmacia) was added at a concentration of 1 unit per milligram of YcjG. The progress of the cleavage reaction was monitored by ESI-MS. When thrombin cleavage of the His tag was complete, the protein was dialyzed against 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5). The cleaved protein was purified over a ResourceQ strong anion-exchange

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; DAP, diaminopimelate; DIEA, *N*,*N*-diisopropylethylamine; Fmoc, fluorenylmethoxycarbonyl; INT, *p*-iodonitrotetrazolium violet; MLE, muconate lactonizing enzyme; MR, mandelate racemase; NAAAR, *N*-acylamino acid racemase; OMe, methyl ester; OSBS, *o*-succinylbenzoate synthase; OtBu, *tert*-butyl ester; PyBOP, benzotriazol-1-yloxytripyrrolidinephosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

column (Pharmacia) using 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5) as the wash buffer and a linear gradient of 0 to 50% NaCl (1 M) over 240 mL in wash buffer. After removal of the 10-histidine tag, the Gly-Ser-His sequence remains at the N-terminus of the protein, giving a final expected mass of 34 955 (observed by ESI-MS as 34 993.9 ± 35).

Cloning, Expression, and Purification of YkfB. The *ykfB* gene from *B. subtilis* strain 168 was PCR-amplified following the procedure described for the *ycjG* gene. However, due to the insolubility of the His-tagged protein, the *ykfB* gene was subcloned into the *Nde*I and *Bam*HI sites of the pET17B vector to produce a protein without an N-terminal His tag. The sequence of the cloned gene was verified by dideoxynucleotide sequencing.

The recombinant plasmid was transformed into BL21(DE3) *E. coli* cells; expression of soluble protein occurred when a 2 L culture of LB was grown without induction at room temperature until an OD_{600} of >2.0 was reached. Cells were harvested and lysed as described for the purification of YcjG. The cell lysate was applied to a DEAE Sepharose Fast Flow column (26 mm × 40 cm) in 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5). The column was washed with 800 mL of 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5), and the protein was eluted with a linear gradient of 0 to 50% NaCl (1 M) over 1600 mL in wash buffer. Fractions containing YkfB were identified by SDS−PAGE analysis, and the purest fractions were pooled and concentrated. The purity of YkfB was greater than 95% as judged by SDS−PAGE. The purified protein was then dialyzed against 10 mM Tris and 5 mM $MgCl_2$ (pH 7.5); long-term storage was in 40% glycerol at −20 °C. The mass of the protein was verified by ESI-MS (expected mass of 39 472.6, observed mass of 39 500.5 ± 39.5).

Cloning, Expression, and Purification of YkfC. The *ykfC* gene from *B. subtilis* was cloned using the same protocol that was described for *ycjG* into the *Nde*I and *Xho*I sites of the modified pET15B expression vector. His-tagged YkfC was expressed and purified following the same procedure described for YcjG. The purified protein was dialyzed against 20 mM Tris and 100 mM KCl (pH 7.5). Storage in 40% glycerol at −20 °C resulted in a significant loss of activity; therefore, dialyzed YkfC was stored at 4 °C without glycerol for up to 48 h without any loss of activity.

Cloning, Expression, and Purification of PepD. The gene encoding peptidase D (PepD) from *E. coli* was cloned using the same protocol. Due to problems expressing the His-tagged protein, the PepD gene was subcloned into pET17B as described for the *ykfB* gene. The soluble protein was expressed in a 2 L culture of LB grown at 37 °C without induction for approximately 30 h. Cells were harvested and lysed as described above, and the protein was purified on a DEAE Sepharose Fast Flow column as described for YkfB. The wash buffer was 20 mM Tris (pH 8.0), and the protein was eluted with a linear gradient of 0 to 50% NaCl (1 M) over 1600 mL in wash buffer. The location of PepD in the eluted fractions was verified by SDS−PAGE. PepD-containing fractions were pooled, concentrated, and stored in 40% glycerol at −20 °C.

^{1}H NMR Assay for Dipeptide Epimerase Activity. Purified YcjG or YkfB was exchanged into D_2O using an Amicon stirred cell. A 10 mL aliquot of protein was concentrated to 2 mL; 8 mL of D_2O was added, and the protein solution was again concentrated to 2 mL. This process was repeated two additional times. Samples for NMR analysis contained 100 mM substrate, 20 mM Tris-HCl, 10 mM $MgCl_2$, and 3−5 mg of protein in D_2O in a 500 μL volume at pH 8. A duplicate sample without enzyme was made for comparison. Samples were incubated overnight at 37 °C.

ESI-MS Assay for Dipeptide Epimerase Activity. Purified YcjG or YkfB was exchanged into D_2O as described above. Samples were made in duplicate so that one could serve as a non-enzyme-containing control. Samples contained each substrate (10 mM), 20 mM ammonium acetate (pH 8), 10 mM $MgCl_2$, and 100 μg of protein in D_2O in a volume of 1 mL. After incubation of both samples at 37 °C from time periods ranging from 1 h to overnight, the protein was removed with a 0.5 mL 10 000 molecular weight cutoff (MWCO) Ultra-Free Biomax protein concentrator (Millipore). The concentrator was prewashed three times with deionized water by spinning it in an Eppendorf microcentrifuge at 8000 rpm for 3 min. Any residual water was removed from the concentrator before application of the sample. The eluted solution of substrates was analyzed directly by ESI-MS, utilizing a cone voltage of 16 V.

Coupled Enzyme Assay for Dipeptide Epimerase Activity. A continuous spectrophotometric coupled enzyme assay was devised for the determination of the dipeptide epimerase activity of YcjG and YkfB. An aliquot of YcjG or YkfB was added to a solution (1.5 mL) containing a dipeptide substrate (for example, L-Ala-D-Glu), 50 mM Tris (pH 8.5), 1.5 mM NAD^+, 1.5 mM *p*-iodonitrotetrazolium violet (INT), 2 units of diaphorase, 7 units of L-alanine dehydrogenase, 67 μM $CoCl_2$, and purified PepD to hydrolyze the epimerized dipeptide (for example, L-Ala-L-Glu). The conversion of INT from the oxidized to the reduced form at 30 °C was monitored by the increase in absorbance at 500 nm (*11*, *12*). The extinction coefficient (ϵ_{500}) of reduced INT was determined to be 12 990 M^{-1} cm^{-1} using diaphorase to oxidize known concentrations of NADH. Determinations of k_{cat} and K_M were performed by varying the concentration of the dipeptide substrate.

Coupled Enzyme Assay for Endopeptidase (YkfC) Activity. To determine kinetic constants for YkfC, the coupled enzyme assay described above was expanded to include YcjG as a coupling enzyme to utilize the L-Ala-D-Glu produced by YkfC from murein peptide analogues L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, L-Ala-γ-D-Glu-L-Lys-D-Ala, or L-Ala-γ-D-Glu-L-Lys. Determinations of k_{cat} and K_M were performed by varying the concentration of the murein peptide analogue.

Synthesis of L-Ala-D-Glu. Two millimoles each of Boc-L-Ala-OH (Advanced ChemTech) and D-Glu(OtBu)-OtBu (Bachem) and 2.2 mmol of PyBOP (Calbiochem) were dissolved in 20 mL of $CHCl_3$. Five millimoles of *N*,*N*-diisopropylethylamine (DIEA) was added, and the solution was stirred for 30 min at room temperature. The solution was then washed five times with 25 mL of phosphate buffer (pH 5.5). The solvent was removed in vacuo. To deprotect the synthesized Boc-L-Ala-D-Glu(OtBu)-OtBu, the residue was dissolved in a minimal amount of CH_2Cl_2, and 5 mL of TFA and 4 mmol of anisole were added. The solution was stirred at room temperature for 4 h. The TFA solution was added to 200 mL of chilled ether, at which point a precipitate formed. The precipitate was collected by filtration. The peptide was dissolved in 25 mL of 10% acetic acid and

washed twice with 10 mL of a 1:1 ethyl acetate/ether mixture, once with 10 mL of CH_2Cl_2, and once with 10 mL of ether. The aqueous phase was lyophilized. The purity of the synthesized dipeptide was judged by ESI-MS (expected mass of 218.2, observed mass of 218.2 + 1). ^{1}H NMR (D_2O): δ 1.32 (d, 3, $-CH_3$), 1.77 (m, 1, $-CHHCH_2COOH$), 1.96 (m, 1, $-CHHCH_2COOH$), 2.09 (m, 2, $-CH_2CH_2COOH$), 3.77 [q, 1, $-CH(CH_3)-$], 4.0 [q, 2, $-CH(CH_2CH_2COOH)-$].

Synthesis of L*-Ala-*D*-Asp*. The coupling of Boc-L-Ala-OH and D-Asp(OMe)-OMe (Bachem) was performed as described for L-Ala-D-Glu. After coupling had been carried out, deprotection of the dimethyl ester was performed by dissolving the residue after solvent removal in 29 mL of methanol and 21 mL of 2 M NaOH. The solution was stirred for 2 h at room temperature. Methanol was removed in vacuo, and the resulting cloudy solution was washed twice with 10 mL of ether. The solution was acidified to pH 2 with 3 M HCl and extracted twice with 25 mL aliquots of ethyl acetate. The organic layer was washed with 25 mL of water and dried, and the solvent was removed in vacuo. To remove the N-terminal Boc protecting group, TFA deprotection was carried out as described for L-Ala-D-Glu. The purity of the L-Ala-D-Asp was judged by ESI-MS (expected mass of 204.2, observed mass of 204.3 + 1). ^{1}H NMR (D_2O): δ 1.11 (d, 3, $-CH_3$), 2.36 (m, 1, $-CHHCOOH$), 2.55 (m, 1, $-CHHCOOH$), 3.36 [q, 1, $-CH(CH_3)-$], 4.26 [q, 1, $-CH(CH_2COOH)-$].

Preparation of L*-Ala-*D*-Met*. L-Ala-D-Met was prepared enzymatically from L-Ala-L-Met according to the following procedure. L-Ala-L-Met (100 mg; Bachem) was dissolved in 15 mL of 50 mM Tris-HCl (pH 8.5). YcjG (100 μg) was added, and epimerization was allowed to occur overnight at 37 °C. The enzyme was removed by filtration through a 0.5 mL protein concentrator as described above for the mass spectral experiments. After removal of YcjG, 2.1 mg of PepD was added, and the reaction mixture was incubated at 37 °C until hydrolysis of the remaining L-Ala-L-Met was complete, approximately 1.5 h. L-Ala-D-Met was purified in 5 μmol increments using a Vydac semipreparative reverse phase HPLC column (buffer A was acetonitrile and buffer B 0.1% TFA in water, flow rate of 1.3 mL/min; elution conditions, 100% B for 2 min, followed by a linear gradient to 100% A over the course of 45 min; under these conditions, L-Ala-D-Met eluted at approximately 24 min). The purity of the L-Ala-D-Met was determined by ^{1}H NMR and ESI-MS (expected mass of 220.3, observed mass of 220.3 + 1). ^{1}H NMR (D_2O): δ 1.08 (d, 3, $-CH_3$), 1.78 (m, 3, $-SCH_3$), 1.95 (m, 2, $-CH_2CH_2SCH_3$), 2.36 (m, 2, $-CH_2CH_2SCH_3$), 3.34 [q, 1, $-CH(CH_3)-$], 4.13 [q, 1, $-CH(CH_2CH_2SCH_3)-$].

Synthesis of Murein Peptide Analogues. The murein peptide analogues L-Ala-γ-D-Glu-Lys-D-Ala-D-Ala, L-Ala-γ-D-Glu-Lys-D-Ala, L-Ala-γ-D-Glu-Lys, L-Ala-γ-L-Glu-Lys-D-Ala-D-Ala, and L-Ala-D-Glu-Lys-D-Ala-D-Ala were synthesized with a Rainin PS3 peptide synthesizer using the standard protocols of the manufacturer for Fmoc protection/Wang resin solid phase peptide synthesis. Wang resins preloaded with the C-terminal amino acids were used; piperidine was used as the deprotectant, and HBTU and NMM were used as activators with DMF as the solvent. Preloaded Fmoc-D-Ala and Fmoc-L-Lys Wang resins were obtained from Calbiochem. Fmoc protected amino acids were obtained from Calbiochem; Fmoc-D-Glu(OH)-OtBu was obtained from Bachem. The only deviation from the standard protocol was an increase in the coupling time from 25 to 40 min for formation of the γ-linkage. The peptides were simultaneously cleaved from the resin and deprotected with TFA using the procedure recommended by the manufacturer. The deprotected peptides were worked up in the same manner as described for L-Ala-D-Glu. The purity of the peptides was ascertained via HPLC and/or ESI-MS. HPLC analysis was performed using a Waters Spherisorb 5 μm, 4.6 mm $\times$ 250 mm analytical cartridge. The elution conditions were the same as described for the semipreparative HPLC purification of L-Ala-D-Met, with a flow rate of 0.5 mL/min. The masses observed by ESI-MS were as follows: 346.3 + 1 for L-Ala-γ-D-Glu-L-Lys (expected, 346.38), 417.3 + 1 for L-Ala-γ-D-Glu-L-Lys-D-Ala (expected, 417.46), 488.3 + 1 for L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (expected, 488.54), 488.4 + 1 for L-Ala-γ-L-Glu-Lys-D-Ala-D-Ala (expected, 488.54), and 488.4 + 1 for L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (expected, 488.54). ^{1}H NMR (D_2O) for L-Ala-γ-D-Glu-L-Lys: δ 1.26 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.33 (d, 3, $-CH_3$), 1.53 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.54 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.66 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.84 (m, 1, $-CHHCH_2COOH$), 1.97 (m, 1, $-CHHCH_2COOH$), 2.20 (t, 2, $-CH_2CH_2COOH$), 2.84 (t, 2, $-CH_2CH_2CH_2CH_2NH_2$), 3.89 [q, 1, $-CH(CH_3)-$], 4.01 [q, 1, $-CH(CH_2CH_2COOH)-$], 4.05 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)-$]. ^{1}H NMR ($D_2O$) for L-Ala-$\gamma$-D-Glu-L-Lys-D-Ala: δ 1.16 [d, 3, $-CH_3$ (L-Ala)], 1.27 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.31 [d, 3, $-CH_3$ (D-Ala)], 1.52 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.60 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.68 (m, 1, $-CHHCH_2CH_2CH_2CH_2NH_2$), 1.81 (m, 1, $-CHHCH_2COOH$), 1.99 (m, 1, $-CHHCH_2COOH$), 2.23 (t, 2, $-CH_2CH_2COOH$), 2.84 (t, 2, $-CH_2CH_2CH_2CH_2NH_2$), 3.80 [q, 1, $-CH(CH_3)-$], 4.01 [q, 1, $-CH(CH_2CH_2COOH)-$], 4.05 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)-$], 4.15 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)$]. ^{1}H NMR ($D_2O$) for L-Ala-$\gamma$-D-Glu-L-Lys-D-Ala-D-Ala (the methyl protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three doublets at 1.18, 1.22, or 1.32 ppm; the α-protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three quartets at 3.82, 3.94, or 4.20 ppm): δ 1.28 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.52 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.62 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.65 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.79 (m, 1, $-CHHCH_2COOH$), 1.98 (m, 1, $-CHHCH_2COOH$), 2.22 (t, 2, $-CH_2CH_2COOH$), 2.84 (t, 2, $-CH_2CH_2CH_2CH_2NH_2$), 4.03 [q, 1, $-CH(CH_2CH_2COOH)-$], 4.10 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)-$]. ^{1}H NMR ($D_2O$) for L-Ala-$\gamma$-L-Glu-L-Lys-D-Ala-D-Ala (the methyl protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three doublets at 1.18, 1.20, or 1.24 ppm; the α-protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three quartets at 3.53, 3.95, or 4.18 ppm): δ 1.27 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.54 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.62 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.65 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.80 (m, 1, $-CHHCH_2COOH$), 1.95 (m, 1, $-CHHCH_2COOH$), 2.23 (t, 2, $-CH_2CH_2COOH$), 2.84 (t, 2, $-CH_2CH_2CH_2CH_2NH_2$), 3.98 [q, 1, $-CH(CH_2CH_2COOH)-$], 4.12 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)-$]. ^{1}H NMR ($D_2O$) for L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (the

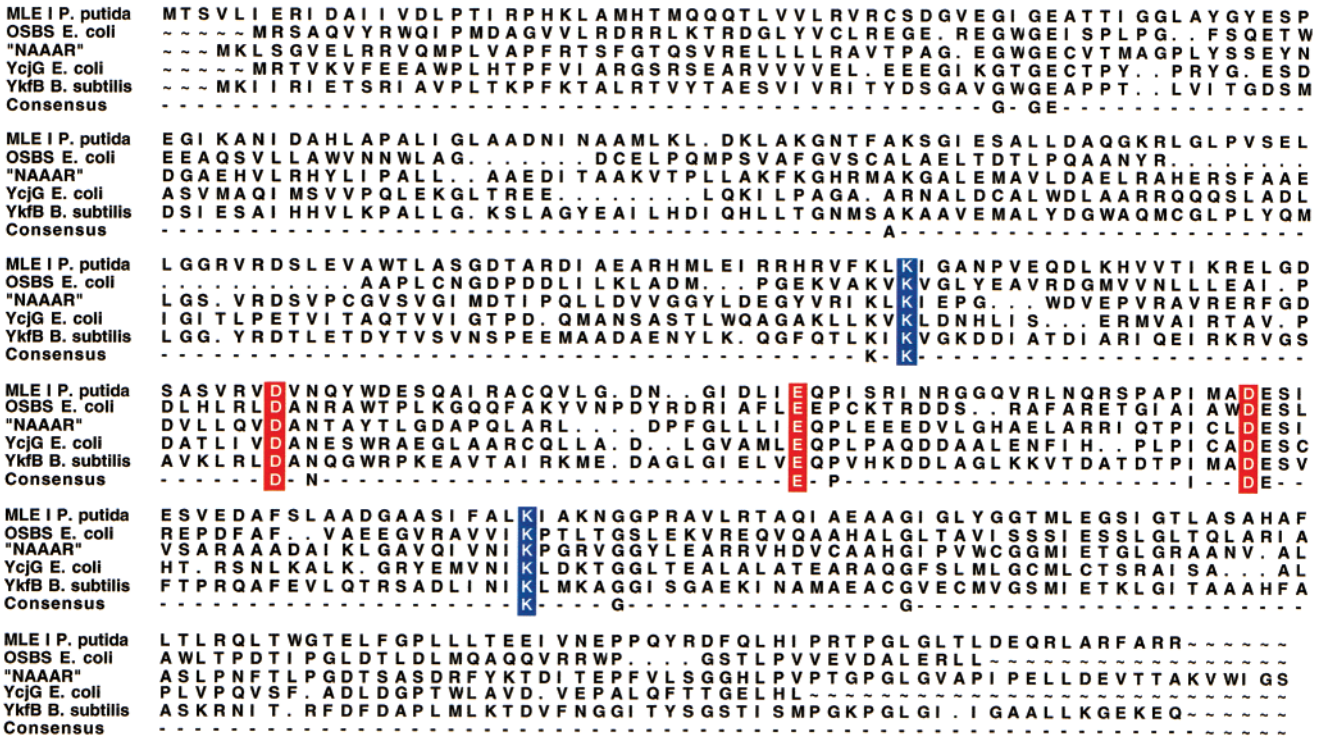

FIGURE 1: Sequence alignment of selected members of the MLE subgroup of the enolase superfamily. Included in the alignment are the amino acid sequences of the muconate lactonizing enzyme (MLE I) from *Pseudomonas putida*, the *o*-succinylbenzoate synthase (OSBS) from *E. coli*, the *N*-acylamino acid racemase/OSBS (NAAAR) from *Amycolaptosis* (*7*), YcjG from *E. coli*, and YkfB from *B. subtilis*. Conserved metal binding residues are shown in red; conserved catalytic residues are shown in blue.

methyl protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three doublets at 1.19, 1.24, or 1.27 ppm; the α-protons of the three alanine residues cannot be assigned unambiguously, and each corresponds to one of three quartets at 3.69, 3.96, or 4.18 ppm): δ 1.30 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.53 (m, 2, $-CH_2CH_2CH_2CH_2NH_2$), 1.63 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.71 (m, 1, $-CHHCH_2CH_2CH_2NH_2$), 1.83 (m, 1, $-CHHCH_2COOH$), 1.87 (m, 1, $-CHHCH_2COOH$), 2.11 (t, 2, $-CH_2CH_2COOH$), 2.85 (t, 2, $-CH_2CH_2CH_2CH_2NH_2$), 4.12 [q, 1, $-CH(CH_2CH_2COOH)-$], 4.17 [q, 1, $-CH(CH_2CH_2CH_2CH_2NH_2)-$].

RESULTS AND DISCUSSION

Identification of Two New Enolase Superfamily Members in E. coli and B. subtilis. PSI-BLAST searches using the NAAAR/OSBS from *Amycolaptosis* sp. (*6*, *7*) as the query sequence revealed two new members of the enolase superfamily, based on the conservation of key active site residues. On the basis of the identities of these active site residues, the two new superfamily members were assigned to the MLE subgroup of the enolase superfamily.

The sequence of YcjG from *E. coli* is 26.6% identical and 34.2% similar to the query sequence. The sequence of YcjG contains the three conserved metal binding ligands (Asp 176, Glu 202, and Asp 225), corresponding to Asp 198, Glu 224, and Asp 249 of MLE, respectively. The two lysine residues characteristic of members of the MLE subgroup are also conserved, Lys 151 (in a Lys 149-X-Lys 151 motif) and Lys 247, corresponding to Lys 169 and Lys 273 of MLE, respectively (*5*).

The sequence of YkfB from *B. subtilis* is 29.1% identical and 40.4% similar to the query sequence. The sequence of YkfB contains the three conserved metal binding ligands (Asp 191, Glu 219, and Asp 244) as well as the two conserved lysine residues, Lys 162 (in a Lys 160-X-Lys 162 motif) and Lys 268.

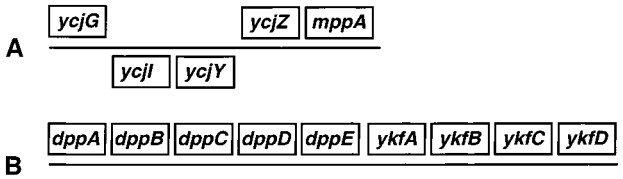

FIGURE 2: Genomic context. The genomic context of the enolase superfamily members encoded by *ycjG* (*E. coli*) and *ykfB* (*B. subtilis*) are represented. *ycjI* and *ycjY* are transcribed in the opposite direction compared to *ycjG*.

The sequences of YcjG and YkfB are 31.9% identical. An alignment of the sequences of several MLE subgroup members, including YcjG and YkfB, is shown in Figure 1. Although YcjG and YkfB were annotated as a "putative muconate cycloisomerase" and a "chloromuconate cycloisomerase homologue", respectively, both proteins were of unknown function.

Genomic Context of ycjG (E. coli). In the *E. coli* genome, immediately downstream of *ycjG* are two genes encoded by the complementary strand, *ycjI* and *ycjY* (Figure 2A). YcjI is 29.4% identical to endopeptidase I of *Bacillus sphaericus*, a metal-dependent enzyme which hydrolyzes the γ-D-Glu-*meso*-DAP bond in the murein peptides L-Ala-γ-D-Glu-*meso*-DAP and L-Ala-γ-D-Glu-*meso*-DAP-D-Ala (*13*). YcjY is most closely related to the C-terminal domains of the dipeptidyl peptidase IV (DPPIV) family of serine proteases, although the Ser-Asp-His catalytic triad is not conserved (*14*, *15*). Further downstream of *ycjG*, *ycjZ*, a putative transcriptional regulator, and *mppA* are transcribed in the same

direction as *ycjG*. MppA is a characterized periplasmic binding protein specific for the import of L-Ala-γ-D-Glu-*meso*-DAP into the cytoplasm (*16*).

Genomic Context of ykfB (B. subtilis). Two operons in the vicinity of *ykfB* provide contextual information (Figure 2B). The first is composed of five genes, *dppA*−*E*. *dppA* has been shown to encode a D-aminopeptidase (*17*). The dipeptide transport operon encoded by *dppB*−*E* (*18*) is homologous to periplasmic binding protein-dependent ABC transport operons. On the basis of sequence homologies, *dppE* is predicted to encode the periplasmic dipeptide binding protein, *dppB* and *dppC* are predicted to encode the permease, and *dppD* is predicted to encode one of two ATP-binding proteins.

Downstream of the operon for *dppA* and *dppC*−*E* is a second operon, comprised of *ykfA*−*D*. YkfA is 36% identical to the microcin C7 immunity factor MccF of *E. coli*; MccF has no known catalytic activity but is responsible for resistance of *E. coli* to exogenous microcin C7 (*19*). Downstream of *ykfB* are *ykfC* and *ykfD*. YkfC is 27.7% identical to the γ-D-glutamyl-L-diamino acid endopeptidase II of *B. sphaericus*, an enzyme which hydrolyzes the same linkage in the murein peptide as does endopeptidase I (*20*). YkfD is 65% identical to OppF, the oligopeptide transport ATP-binding protein of *B. subtilis*. Since the canonical composition of periplasmic binding protein-dependent ABC transporters includes two membrane-associated subunits harboring ATP-binding cassettes, it is possible that YkfD may be the complement to DppD in the upstream dipeptide transport operon.

Identification of a Possible Function for YcjG and YkfB. Two sets of clues suggested the functions of YcjG and YkfB. The first was provided by the paradigm of the enolase superfamily. All members of the superfamily characterized to date catalyze reactions initiated by abstraction of an α-proton to form an enolate anion intermediate, so it was expected that YcjG and YkfB would do the same. The second clue was provided by the genomic context of the genes encoding these proteins. In both cases, the genomic context indicated a possible role in the metabolism of the murein peptide of peptidoglycan. The myriad of murein hydrolases known to exist degrade peptidoglycan only to the final dipeptide L-Ala-D-Glu. No known bacterially encoded peptidase can cleave this dipeptide. However, if L-Ala-D-Glu could be epimerized to L-Ala-L-Glu, hydrolysis could occur with known dipeptidases. In addition, such a dipeptide epimerization reaction could proceed through a mechanism initiated by abstraction of the α-proton of the D-glutamate residue. Therefore, it was hypothesized that YcjG and YkfB catalyze the epimerization of L-Ala-D-Glu to L-Ala-L-Glu.

Assaying Dipeptide Epimerase Activity of YcjG and YkfB. A simple qualitative assay was used first to determine whether YcjG and/or YkfB could epimerize commercially available L-Ala-L-Glu. L-Ala-L-Glu was presented to each of the two enzymes in D_2O. On the basis of the homology to other members of the enolase superfamily, a dipeptide epimerization catalyzed by YcjG or YkfB would take place by a two-base mechanism, in which one of the conserved lysines would act as a general base, abstracting a proton, and the other lysine would act as a general acid, protonating the enolate anion intermediate. If epimerization occurred, the α-proton would be abstracted by the general base and replaced with deuterium from the general acid. ^{1}H NMR spectroscopy was used to determine if deuterium exchange had occurred by examining the disappearance of the glutamate α-proton. Disappearance of the α-proton of the glutamate residue of L-Ala-L-Glu was observed in the presence of either YcjG or YkfB (Figure 3). The formation of a diastereomeric mixture was further confirmed by the appearance of a new quartet at approximately 3.64 ppm and a new doublet at approximately 1.25 ppm (Figure 3B), due to the L-alanine α-proton and methyl protons, respectively, of the newly formed L-Ala-D-Glu dipeptide. These results indicated that YcjG and YkfB could catalyze the epimerization of L-Ala-L/D-Glu.

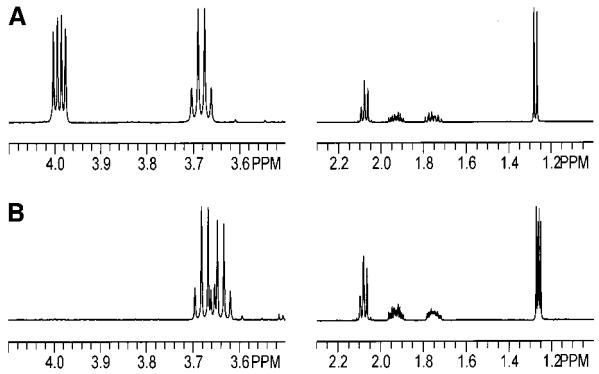

FIGURE 3: ^{1}H NMR assay for dipeptide epimerase activity. ^{1}H NMR spectra of L-Ala-L-Glu in the absence (A) and presence (B) of YcjG or YkfB. The signal for the α-proton of the glutamic acid residue of L-Ala-L-Glu is at approximately 4.0 ppm and is absent in spectrum B due to the incorporation of deuterium at the α-proton position.

In subsequent studies, experiments performed in D_2O were analyzed using electrospray ionization mass spectrometry (ESI-MS) to detect an increase in mass of the substrates as deuterium was incorporated. Using this approach, we were able to analyze a large number of dipeptides in mixtures. All of the L-Ala-L-X dipeptides (where X was any amino acid except Cys), L-Lys-L-Ala, D-Ala-D-Ala, L-Glu-L-Glu, Gly-L-Glu, L-Lys-L-Glu, L-Phe-L-Glu, L-Pro-L-Glu, and L-Ser-L-Glu were assayed in different combinations. L-Lys-L-Ala and D-Ala-D-Ala were chosen because they represent components of the murein peptide. The L-X-L-Glu peptides were chosen to determine if L-Ala was required in the first position of the dipeptide and, also, to sample a range of charges, sizes, and hydrophobicities in the first position. The results of these experiments are shown in Table 1.

Neither enzyme could epimerize L-Ala-L-Arg, L-Ala-L-Lys, or L-Ala-L-Pro; L-Ala-L-His was epimerized by YcjG (but not YkfB) at pH 8 but not at pH 6. This would indicate that either a positive charge or the cyclic structure of proline in the second position is incompatible with the active site architectures. In addition, neither enzyme was able to epimerize L-Glu-L-Glu, L-Lys-L-Glu, L-Lys-L-Ala, or D-Ala-D-Ala, indicating that the presence of a positively or negatively charged or D-amino acid residue in the first position of the dipeptide is not acceptable to either enzyme.

YcjG has a broader substrate specificity than YkfB. YcjG was able to epimerize all of the L-Ala-L-X dipeptides assayed by the mass spectral approach except those excluded above, while YkfB was only able to epimerize L-Ala-L-Glu, L-Ala-L-Asp, L-Ala-L-Ala, L-Ala-L-Leu, and L-Ala-L-Ser. YcjG was

Table 1: Substrate Specificities of YcjG and YkfB

dipeptide	YcjG (*E. coli*)	YkfB (*B. subtilis*)	dipeptide	YcjG (*E. coli*)	YkfB (*B. subtilis*)
Ala-Glu	X[a]	X	Ala-Ser	X	X
Ala-Ala	X	−[b]	Ala-Thr	X	−
Ala-Asn	X	−	Ala-Trp	X	−
Ala-Asp	X	X	Ala-Tyr	X	−
Ala-Arg	−	−	Ala-Val	X	−
Ala-Gln	X	−	Glu-Glu	−	−
Ala-Gly	X	−	Gly-Glu	X	−
Ala-His	X	−	Lys-Glu	−	−
Ala-Ile	X	−	Phe-Glu	X	−
Ala-Leu	X	X	Pro-Glu	−	X
Ala-Lys	−	−	Ser-Glu	X	X
Ala-Met	X	X	Lys-Ala	−	−
Ala-Phe	X	−	D-Ala-D-Ala	−	−
Ala-Pro	−	−			

[a] Full epimerization of substrate by enzyme in an overnight incubation. [b] Epimerization of substrate did not occur.

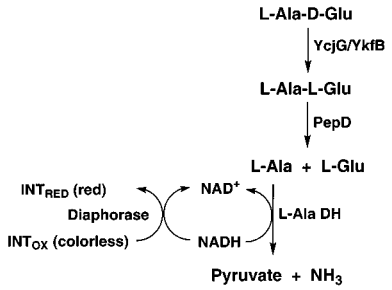

FIGURE 4: Coupled enzyme assay for dipeptide epimerase activity. A diagram of the spectrophotometric coupled enzyme assay used to monitor dipeptide epimerase activity, using L-Ala-D-Glu as a representative substrate for YcjG or YkfB.

active with L-Gly-L-Glu, L-Phe-L-Glu, and L-Ser-L-Glu; YkfB was active with L-Ser-L-Glu and, oddly, L-Pro-L-Glu. In either case, when multiple dipeptide substrates were present, only L-Ala-L-Glu was fully epimerized after an abbreviated incubation period. In addition, neither enzyme was able to catalyze the OSBS reaction or the NAAAR reaction using *N*-acetyl-L-methionine as the substrate (data not shown).

Kinetic Parameters for YcjG and YkfB. A continuous spectrophotometric coupled-enzyme assay was devised to quantitate the dipeptide epimerase activity of YcjG and YkfB with various dipeptide substrates at pH 8.5 (Figure 4). Four substrates were assayed: L-Ala-D-Glu, L-Ala-D-Asp, L-Ala-D-Gln, and L-Ala-D-Met. After epimerization by YcjG or YkfB, the L-Ala-L-X dipeptide that formed was hydrolyzed to amino acids by the action of PepD from *E. coli*, a broad specificity dipeptidase (*21*). PepD has no activity with L-Ala-D-Glu and L-Ala-D-Asp. However, PepD exhibited slight activity toward L-Ala-D-Gln and L-Ala-D-Met; the rate for this activity could be reliably subtracted from the overall rate to give the rate for the dipeptide epimerase-catalyzed reaction. The subtraction was performed by measuring the steady-state rate of L-Ala-D-Gln or L-Ala-D-Met consumption by PepD before the addition of YcjG or YkfB; average values for the background activity were 15% for L-Ala-D-Gln and 28% for L-Ala-D-Met. PepD was significantly active with L-Ala-D-Ala, which precluded its use as a substrate in this assay. The L-Ala produced from the L-Ala-L-X dipeptides by the action of PepD was converted to pyruvate and ammonia by the action of L-alanine dehydrogenase from *B. subtilis* with the concomitant formation of NADH from NAD^+. Deamination of L-Ala is not favored at the pH of this assay, so diaphorase from *Clostridium kluyveri* was used to drive the reaction to completion. Diaphorase catalyzes the transfer of electrons from NADH to a dye [in this case, *p*-iodonitrotetrazolium violet (INT)] to produce NAD^+.

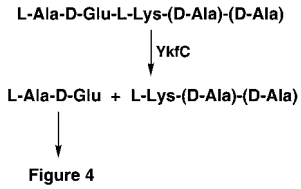

FIGURE 5: Coupled enzyme assay for endopeptidase activity. A diagram of the spectrophotometric coupled enzyme assay used to monitor the endopeptidase activity catalyzed by YkfC. Penta-, tetra-, and tripeptides are used as substrates to produce L-Ala-D-Glu, the formation of which can be monitored as shown in Figure 4.

The kinetic parameters determined for YcjG and YkfB are shown in Table 2. YkfB shows no activity with L-Ala-D-Gln, so no kinetic parameters for this dipeptide are shown in the table. Both enzymes catalyze the epimerization of L-Ala-D-Glu with k_{cat}/K_M values in the range of 10^4 M^{-1} s^{-1}; however, at high substrate concentrations, YkfB experiences substrate inhibition while YcjG does not. Activity with L-Ala-D-Asp by YcjG was comparable to that with L-Ala-D-Glu, but substrate inhibition at concentrations only slightly above K_M allows L-Ala-D-Glu to be used preferentially in assays where both substrates were present. Activity with L-Ala-D-Asp by YkfB was significantly decreased relative to that of L-Ala-D-Glu with a k_{cat} value of only 0.053 ± 0.009 s^{-1} compared with a value of 15 ± 2.7 s^{-1} for L-Ala-D-Glu; YkfB was also inhibited by higher concentrations of L-Ala-D-Asp with a K_I value of $(1.1 \pm 0.5) \times 10^{-3}$ M. Both enzymes exhibited 10-fold decreases in k_{cat}/K_M with L-Ala-D-Met as the substrate; YcjG was subject to substrate inhibition at higher concentrations. While YkfB had no activity on L-Ala-D-Gln, YcjG showed a 10-fold decrease in catalytic efficiency, with no substrate inhibition. For either enzyme, substrate inhibition was most significant with substrates other than L-Ala-D-Glu.

Endopeptidase Activity of YkfC (B. subtilis). The coupled enzyme assay used to determine the kinetic parameters for YcjG and YkfB was modified (Figure 5) to measure the ability of YkfC to hydrolyze the the γ-D-Glu-L-Lys bond in murein peptide analogues L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala,

Table 2: Kinetic Parameters for the Reactions Catalyzed by YcjG and YkfB

	YcjG (*E. coli*)				YkfB (*B. subtilis*)			
substrate	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_I (M)	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_I (M)
L-Ala-D-Glu	$(1.3 \pm 0.3) \times 10^{-4}$	10 ± 0.4	7.7×10^4	−	$(3.2 \pm 1.1) \times 10^{-4}$	15 ± 2.7	4.7×10^4	2.0×10^{-3}
L-Ala-D-Asp	$(1.9 \pm 0.7) \times 10^{-4}$	17 ± 2.7	8.9×10^4	8.2×10^{-4}	$(2.8 \pm 1.2) \times 10^{-5}$	0.053 ± 0.009	1.9×10^3	1.1×10^{-3}
L-Ala-D-Met	$(6.9 \pm 0.4) \times 10^{-4}$	1.9 ± 0.1	2.8×10^3	2.1×10^{-3}	$(5.1 \pm 1.7) \times 10^{-4}$	1.1 ± 0.1	2.2×10^3	−
L-Ala-D-Gln	$(1.8 \pm 0.2) \times 10^{-3}$	3.3 ± 0.1	1.8×10^3	−	−	−	−	−

Table 3: Kinetic Constants for Reactions Catalyzed by YkfC

substrate	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
L-Ala-D-Glu-L-Lys-D-Ala-D-Ala	$(3.1 \pm 0.4) \times 10^{-4}$	5.7 ± 0.3	1.8×10^4
L-Ala-D-Glu-L-Lys-D-Ala	$(1.2 \pm 0.1) \times 10^{-4}$	2.6 ± 0.1	2.2×10^4
L-Ala-D-Glu-L-Lys	$(2.9 \pm 0.5) \times 10^{-4}$	1.2 ± 0.1	4.1×10^3

L-Ala-γ-D-Glu-L-Lys-D-Ala, and L-Ala-γ-D-Glu-L-Lys. Hydrolysis releases L-Ala-D-Glu, which can be monitored by addition of YcjG to the coupled assay to convert L-Ala-D-Glu to L-Ala-L-Glu. The kinetic parameters determined for YkfC are shown in Table 3. Although k_{cat} was slightly larger for the pentapeptide substrate, YkfC had similar activity with the pentapeptide and tetrapeptide. The catalytic efficiency was decreased 10-fold with the tripeptide relative to that with the penta- and tetrapeptides. In addition, YkfC showed no hydrolytic activity on peptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala or L-Ala-γ-L-Glu-L-Lys-D-Ala-D-Ala, confirming that YkfC is specific for the γ-D-Glu-L-Lys peptide bond. Since the homology between YkfC and endopeptidase II was part of the basis for the functional assignment of the dipeptide epimerases YcjG and YkfB, the ability of YkfC to hydrolyze these peptides specifically to yield L-Ala-D-Glu lends support to that assignment.

YcjI and YcjY. As described above, YcjI and YcjY are encoded by genes proximal to YcjG but transcribed in the opposite direction. These genes were cloned, and the proteins were purified. It was hypothesized that YcjI might be able to hydrolyze the same bond as YkfC, and that YcjY might hydrolyze some other bond in the murein peptide. However, we were unable to demonstrate any activity of either enzyme on the synthesized murein peptide analogues. In Gram-negative bacteria like *E. coli*, L-Lys is absent in the murein peptide, so the enzymes in *E. coli* which hydrolyze peptide bonds in the murein peptide might be specific for *meso*-DAP and unable to hydrolyze peptides containing Lys.

Identification of L-Ala-D/L-Glu Epimerase Homologues. Homologues of YkfB have been identified in several other organisms: *Bacillus halodurans*, *Clostridium acetobutylicum*, *Clostridium difficile*, and *Thermotoga maritima*. The enzymes from *C. acetobutylicum* and *T. maritima* have been purified and shown to catalyze epimerization of dipeptides. The enzyme from *C. acetobutylicum* epimerizes L-Ala-D-Glu and L-Ala-D-Asp; the enzyme from *T. maritima* epimerizes L-Ala-D-Glu, L-Ala-D-Met, and L-Ala-D-Ala. The identification of four enzymes from four different organisms that catalyze the dipeptide epimerase reaction further supports this functional assignment. Despite the widely differing substrate specificities of each enzyme, all catalyze the epimerization of L-Ala-D-Glu.

Physiological Role of L-Ala-D/L-Glu Epimerase Activity. Because all of the characterized dipeptide epimerases share L-Ala-D-Glu as a common substrate, a role in murein peptide metabolism is likely. This conclusion is supported by the genomic context of the *ycjG* and *ykfB* genes. Under what conditions would L-Ala-D/L-Glu epimerase be necessary? Recycling of peptidoglycan has been shown in *E. coli* but not in *B. subtilis*, and it is believed that peptidoglycan is broken down only as far as the tripeptide L-Ala-D-Glu-*meso*-DAP (*22*). This tripeptide is then directly used in the formation of new peptidoglycan. A role for L-Ala-D/L-Glu epimerase in recycling of the murein sacculus is therefore unlikely. Both Gram-negative and Gram-positive bacteria release components of peptidoglycan to their surroundings during cell growth. Under nutrient-limiting conditions, such as stationary phase, L-Ala-D/L-Glu epimerase could function to convert L-Ala-D-Glu in the medium to L-Ala-L-Glu, which would allow the dipeptide to be further metabolized. Because L-Ala-D/L-Glu epimerases have not been described previously, these enzymes likely are produced under a highly specific set of conditions.

CONCLUSION

We have identified two members of the enolase superfamily, proteins of previously unknown function encoded by the *E. coli* and *B. subtilis* genomes, as L-Ala-D/L-Glu epimerases, which catalyze the epimerization of a component of the murein peptide. These functional assignments were accomplished by incorporating the catalytic paradigm of the enolase superfamily into an analysis of the genomic context of each of the encoding genes; neither factor alone would have allowed identification of function. The L-Ala-D/L-Glu epimerase reaction expands the repertoire of reactions catalyzed by members of the enolase superfamily and highlights the ability of Nature to utilize existing structure and function relationships in the evolution of new activities for changing metabolic contexts. Finally, given the ability of the OSBS from *Amycolaptosis* to catalyze a promiscuous NAAAR reaction, the identification of L-Ala-D/L-Glu epimerase lends support to the proposal that new enzymes evolve by duplication of the gene encoding an enzyme that catalyzes low levels of the desired new reaction followed by optimization of the new reaction in response to adaptive pressure (*8*).

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