Conditionally Lethal *Escherichia coli* Murein Mutants Contain Point Defects That Map to Regions Conserved among Murein and Folyl Poly-γ-glutamate Ligases: Identification of a Ligase Superfamily

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ABSTRACT: Bacterial peptidoglycan biosynthesis includes four enzymatic reactions in which successive amino acid residues are ligated to uridine diphospho-*N*-acetylmuramic acid (UDP-MurNAc). By comparing the amino acid sequences of MurC, -D, -E, and -F proteins from various bacterial genera, four regions of homology were identified. A profile search of Swissprot for related sequences revealed that these regional similarities were present in the folyl-γ-polyglutamate ligases. These sequence homologies appear to track with catalytic function: both enzyme families proceed through an ordered kinetic mechanism and form product via an acyl phosphate intermediate. Two highly conserved residues in region II were examined through site-directed mutagenesis of the murein D-alanyl-D-alanine-adding enzyme from *Escherichia coli* (*murF*; E158 and H188). All mutations were highly detrimental to activity with enzyme specific activity reductions of 200−4500-fold, validating the critical nature of these residues. DNA sequence analysis from three *E. coli* mutants harboring the *murC3* (G344D), *murE1* (G344K, A495S), and *murF2* (A288T) mutations revealed the presence of point mutation(s) closely associated with the fourth of these aligned regions. The *murF2* allele, expressed and purified as a glutathione S-transferase::MurF2 fusion, was 181-fold less catalytically active at 30 °C and was further reduced at the nonpermissive temperature (42 °C). Thus the *murF2* temperature-sensitive phenotype arises from a point mutation within a highly conserved region within this protein family. These data argue that these proteins comprise a superfamily of three substrate amide ligases that share significant structural and catalytic homologies.

Both Gram-positive and Gram-negative bacteria synthesize peptidoglycan, a network of glycan strands connected by short, unusual peptide cross-links. A large polymer synthesized from disaccharide pentapeptide monomers, this single molecule surrounds and shapes the bacterial cell, containing the outward forces generated in maintaining an osmotic pressure gradient against the environment (Park, 1996). The biosynthesis of peptidoglycan starts in the cytoplasm with the condensation of phosphoenolpyruvate and UDP-*N*-acetylglucosamine catalyzed by MurA and finishes across the plasma membrane in the periplasm with the transglycosylation and transpeptidation of the disaccharide pentapeptide monomers by the penicillin binding proteins. The focus of the cytoplasmic steps is construction of the pentapeptide side chain by stepwise addition of amino acids to the lactyl carboxylate of UDP-*N*-acetylmuramic acid (van Heijenoort, 1996). These ATP-dependent peptide-bond-forming reactions are catalyzed by MurC, -D, -E, and -F. In *Escherichia coli*, the isolation of conditional lethal alleles for each of the genes encoding these enzymes, many of which are clustered in a large operon at the minute 2 region (Yura et al., 1992), has demonstrated that they are essential for cell viability (Lugtenberg et al., 1972; Lugtenberg & van Schijndel-van Dam, 1972; Miyakawa et al., 1972) and, as such, are attractive targets for antibacterial chemotherapy. A similar gene clustering has also been observed in the Gram-positive organism *Bacillus subtilis* (Errington, 1993). Several examples of each *murC*, *-D*, *-E*, and *-F* gene have been reported from other bacterial genera (Figure 1).

We have reexamined the primary sequence similarities between these *mur* gene products as well as extended the search for homologies to other known protein sequences. This report describes a specific set of localized identities conserved among the murein gene products that are also largely present in certain other ATP-dependent, three-substrate peptidyl ligases, notably the folyl-γ-polyglutamate ligases. Site-directed mutagenesis experiments targeting a conserved Glu and His residue within the second homologous region of the *E. coli* D-Ala-D-Ala[1]-adding enzyme revealed a strong intolerance to even modest substitutions, consistent with a conserved residue essential to either catalysis or folding. DNA sequence determination of temperature-sensitive *E. coli murC*, *-E*, and *-F* alleles localized the genetic defects in each case to the fourth of these homologous regions, highlighting the region's importance to the proper functioning of the protein. Further, expression and activity measurements of the *murF2* allele of the *E. coli* D-Ala-D-Ala-adding enzyme confirmed in vitro the temperature-sensitive defect harbored by this allele relative to the wild type. Thus the *murF2* temperature-sensitive phenotype can be traced directly to a point mutation within a highly conserved region among these proteins. The combined data support the validity of these alignments to predict critical regions of these proteins

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[1] Abbreviations: D-Ala-D-Ala, D-alanyl-D-alanine; PCR, polymerase chain reaction; GST::MurF, glutathione S-transferase::D-alanyl-D-alanine-adding enzyme fusion protein; UDP-MurNAc, uridine diphospho-*N*-acetylmuramic acid; tripeptide, L-Ala-γ-D-Glu-*meso*-A_2pm; pentapeptide, L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala.

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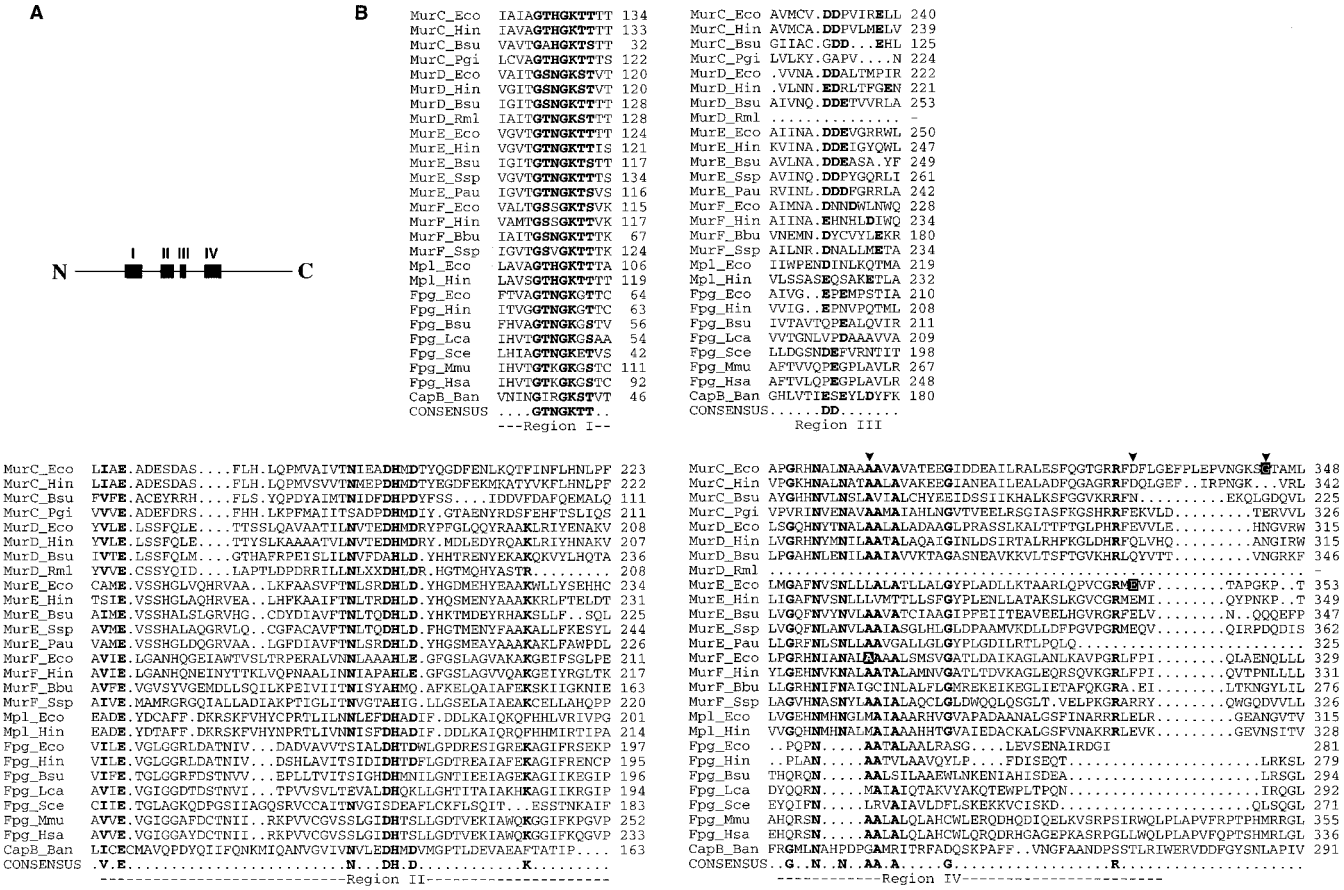

FIGURE 1: Alignment of the inferred amino acid sequences of murein genes *murC*, *-D*, *-E*, and *-F* with the folyl-γ-polyglutamate ligases, murein peptide ligases, and CapB protein. Panel A: Relative placement of regions I−IV within the aligned sequences. Panel B: Regional sequence alignments. Numbers refer to the C-terminal amino acid of each sequence as they are aligned. Long regions of sequence with little homologous information are omitted. Selected residues in boldface type are either invariant or are restricted to a very limited number of highly similar or isosteric replacements. Arrowheads above region IV correspond to the site of point mutations (darkened squares) described in Table 4. Abbreviations and accession numbers are as indicated below; protein sequences were either obtained directly or translated from the sources indicated. MurC_Eco, *Escherichia coli*, X55034; MurC_Hin, *Haemophilus influenzae*, L45775; MurC_Bsu, *Bacillus subtilis*, L31845; MurC_Pgi, *Porphyromonas gingivalis*, D28916; MurD_Eco, *Escherichia coli*, X55034; MurD_Hin, *Haemophilus influenzae*, L45772; MurD_Bsu, *Bacillus subtilis*, Z15056; MurD_Rme, *Rhizobium meliloti*, L25875; MurE_Eco, *Escherichia coli*, X55034; MurE_Hin, *Haemophilus influenzae*, L45769; MurE_Bsu, *Bacillus subtilis*, Z15056; MurE_Ssp, *Synechocystis* sp., D64006; MurE_Pau, *Pseudomonas aeruginosa*, X84053; MurF_Eco, *Escherichia coli*, U67891; MurF_Hin, *Haemophilus influenzae*, L45770; MurF_Bbu, *Borrelia burgdorferi*, U43739; MurF_Ssp, *Synechocystis* sp., X62437. Murein peptide ligases: Mpl_Eco, *Escherichia coli*, P37773; Mpl_Hin, *Haemophilus influenzae*, P43948. Folyl-γ-polyglutamate synthetases: Fpg_Eco, *Escherichia coli*, P08192; Fpg_Hin, *Haemophilus influenzae*, P43775; Fpg_Bsu, *Bacillus subtilis*, Q05865; Fpg_Lca, *Lactobacillus casei*, P15925; Fpg_Hsa, *Homo sapiens*, Q05932; Fpg_Mmu, *Mus musculus* (mouse), P48760; Fpg_Sce, *Saccharomyces cerevisiae*, P36001. CapB protein: CapB_Ban, *Bacillus anthracis*, P19580.

which, by extension, comprise a superfamily of three substrate amide ligases that share significant structural and catalytic homologies. These results supply the basis for highly directed studies relating to active-site structure, mechanism, and function in this broader enzyme family.

EXPERIMENTAL PROCEDURES

Sequence Analysis. All sequence comparisons and manipulations were performed using the GCG package version 8.1 (Wisconsin Sequence Analysis Package by Genetics Computer Group, Inc.). Murein sequences were identified by separate TFASTA search (Pearson & Lipman, 1988) of GenBank with the *E. coli* version of each murein gene, *murC*, *murD*, *murE*, and *murF*. The recovered DNA sequences were translated to peptide sequences and aligned as a single group using the PILEUP algorithm (Feng & Doolittle, 1987). Further database homologies were identified using PROFILEMAKE and PROFILESEARCH from this alignment (Gribskov et al., 1987). Final alignment refinements were made by hand.

Bacterial Strains. All strains used were of *E. coli*. Strain BL21 was purchased from Novagen, and XL-1 Blue (*recA*$^-$) was obtained from Stratagene. *E. coli* strains harboring mutations in various murein genes were obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT. These were strain ST222, CGSC #5988 *murC3* (Miyakawa et al., 1972); strain TKL11, CGSC #5989 *murE1* (Lugtenberg et al., 1972); strain TKL46, CGSC #5990 *murF2* (Lugtenberg & van Schijndel-van Dam, 1972). Plasmid pGEX-KT has been described (Hakes & Dixon, 1992).

Site-Directed Mutagenesis of pGEXMurF. Site-directed mutagenesis was performed directly on plasmid pGEXmurF encoding the *E. coli* UDP-MurNAc-tripeptide:D-alanyl-D-alanine-adding enzyme in fusion with glutathione S-transferase (Anderson et al., 1996). Mutations were introduced using the primer extension, *Dpn*I digestion approach of Stratagene's Quikchange site-directed mutagenesis kit (Papworth et al., 1996). Oligonucleotide primer pairs used to create each mutant are listed in Table 1. Candidate mutant genes were sequenced in order to verify the presence of the

Table 1: Primers Used for Site-Directed Mutagenesis of pGEXMurF[a]

mutation	primer DNA sequence
E158A	5'-ACG CAG TTA TTG CAC TTG GCG CGA AC-3'
	5'-GTT CGC GCC AAG TGC AAT AAC TGC GT-3'
E158G	5'-ACG CAG TTA TTG GAC TTG GCG CGA AC-3'
	5'-GTT CGC GCC AAG TCC AAT AAC TGC GT-3'
E158D	5'-ACG CAG TTA TTG ATC TTG GCG CGA AC-3'
	5'-GTT CGC GCC AAG ATC AAT AAC TGC GT-3'
H188A	5'-CTG GCA GCG GCG GCT CTG GAA GGT TTT GGC-3'
	5'-GCC AAA ACC TTC CAG AGC CGC CGC TGC CAG-3'
H188G	5'-CTG GCA GCG GCG GGT CTG GAA GGT TTT GGC-3'
	5'-GCC AAA ACC TTC CAG ACC CGC CGC TGC CAG-3'
H188D	5'-CTG GCA GCG GCG GAT CTG GAA GGT TTT GGC-3'
	5'-GCC AAA ACC TTC CAG ATC CGC CGC TGC CAG-3'
H188N	5'-CTG GCA GCG GCG AAT CTG GAA GGT TTT GGC-3'
	5'-GCC AAA ACC TTC CAG ATT CGC CGC TGC CAG-3'

[a] These primer pairs are complementary and confer the base pair changes underlined.

desired mutation without further alterations. In each case, a verified mutant plasmid was freshly transformed into strain BL21 for expression.

Expression and Purification of GST::MurF Mutant Alleles. The allelic variants created by site-directed mutagenesis were expressed and purified as described previously for the wild-type GST::MurF protein (Anderson et al., 1996).

*Assay of UDP-MurNAc-tripeptide:*D-*alanyl*-D-*alanine-Adding Enzyme*. Assays were performed by monitoring production of ADP from ATP spectrophotometrically using a coupled assay system of pyruvate kinase and lactate dehydrogenase as described previously (Anderson et al., 1996).

Assays of the site-directed mutant GST::MurF proteins were performed in parallel with a control containing only ATP as substrate. At very high levels of protein, a general ATPase activity was observable in all purified mutants that was not dependent on the presence of other substrates and did not vary in specific activity from mutant to mutant. Wild-type D-Ala-D-Ala-adding enzyme activity measured with all substrates present was 500-fold greater than this "general ATPase" activity. This velocity was deemed background and was subtracted from that seen in the presence of the other two substrates. All measurements of mutant residual activity were performed in triplicate and were reproducible to within 5%.

Cloning and Sequencing of Mutant mur Alleles from E. coli Strains. In order to gain additional structure−function information, the mutant alleles of several *mur* genes were retrieved from the corresponding *E. coli* mutants (Lugtenberg and van Schijndel-van Dam, 1972) using PCR. Primers were designed to retrieve several hundred base pairs of flanking DNA to either side of the reading frame. These primer pairs were as follows: for *murC3*, 5′ primer 5′-CAA TCC GTT GAA CAG GCG-3′ and 3′ primer 5′-CTT CAC GCA GTC CGG CTA AC-3′; for *murE1*, 5′ primer 5′-ATT AAA GGC TAT CGT ATC GCC-3′ and 3′ primer 5′-GCG TAT CCT TGA CGA TTA AC-3′; for *murF2*, 5′ primer 5′-GGT AAG CGT CCA CTG ATG G-3′ and 3′ primer 5′-TGA GCA ATC ATA CGC GGG C-3′. Unique fragments of the anticipated size were retrieved in reactions utilizing 100 ng of genomic DNA template and the appropriate primers. PCR was performed in a Perkin-Elmer Cetus thermocycler using pfu polymerase in buffer supplied by the manufacturer (Stratagene). Conditions of PCR were as follows: for *murC3* and *murE1*, 35 cycles of 97 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 2 min (extension); for *murF2*, 35 cycles of 97 °C for 30 s (denaturation), 56 °C for 30 s (annealing), and 72 °C for 2 min (extension). After this, all PCR reactions were polished at 72 °C for 10 min. The *murF2* allelic PCR product was cleaned using the Wizard PCR Preps DNA purification system (Promega) and ligated into pCR-Script SK(+) (Stratagene) according to the manufacturer's instructions. The *murC3* and *murE1* PCR products were tailed with a single 3′ A by incubation of the completed reactions with 1 unit of Taq polymerase for 9 min at 72 °C. The products were then ligated into pCR 2.1 (Invitrogen). A clone representing each PCR reaction was sequenced by Dye terminator technology (Perkin-Elmer Cetus) on an automated DNA analyzer (Applied Biosystems). In all cases, each pair of clones generated from parallel PCR reactions yielded identical sequence indicating a lack of PCR-generated sequence variants.

RESULTS

Compilation and Alignment of Murein Ligases and Related Protein Sequences. Ikeda and co-workers had previously compared the inferred protein sequences of the four *E. coli* murein genes, *murC*, *-D*, *-E*, and *-F* (Ikeda et al., 1990). Their analysis revealed a highly conserved GXXGK(T/S)-(T/S) motif corresponding to a typical nucleotide fold presumed to be involved in binding the substrate ATP. A second conserved region consisting of generally hydrophobic amino acids was also observed. We have reinvestigated this alignment in light of the increased number of murein gene sequences now available through expanded genomic sequencing efforts. These sequences were derived from a wide range of bacterial genera. Using a FASTA search protocol (Pearson & Lipman, 1988), 17 sequences were identified as homologs of the *murC*, *-D*, *-E*, and *-F* genes. In addition, the highly homologous murein peptide ligase recently described from *E. coli* and a presumptive reading frame encoding this function from *Haemophilus influenzae* were added to the alignment (Mengin-Lecreulx et al., 1996). As shown in Figure 1, alignment of these proteins reinforces the existence of both the highly invariant nucleotide fold (region I) near murein amino acids 120 and the hydrophobic patch (region IV) near murein amino acids 300. However, our more comprehensive lineup better defines region IV, sharpening its description from one of general hydrophobicity to that of a specific consensus sequence: GxxNxxNxxAAxA-5x-G-18x-R.

With more sequences available for comparison, new relationships became apparent. Region II is an extended, previously unrecognized domain in the middle of the protein that contains an invariant glutamate and a highly conserved histidine in mid-protein (Figure 1). This histidine lies between two acidic amino acids nested within a generally hydrophobic region. Curiously, the rhizobial MurD protein sequence fell grossly out of step with the consensus sequence

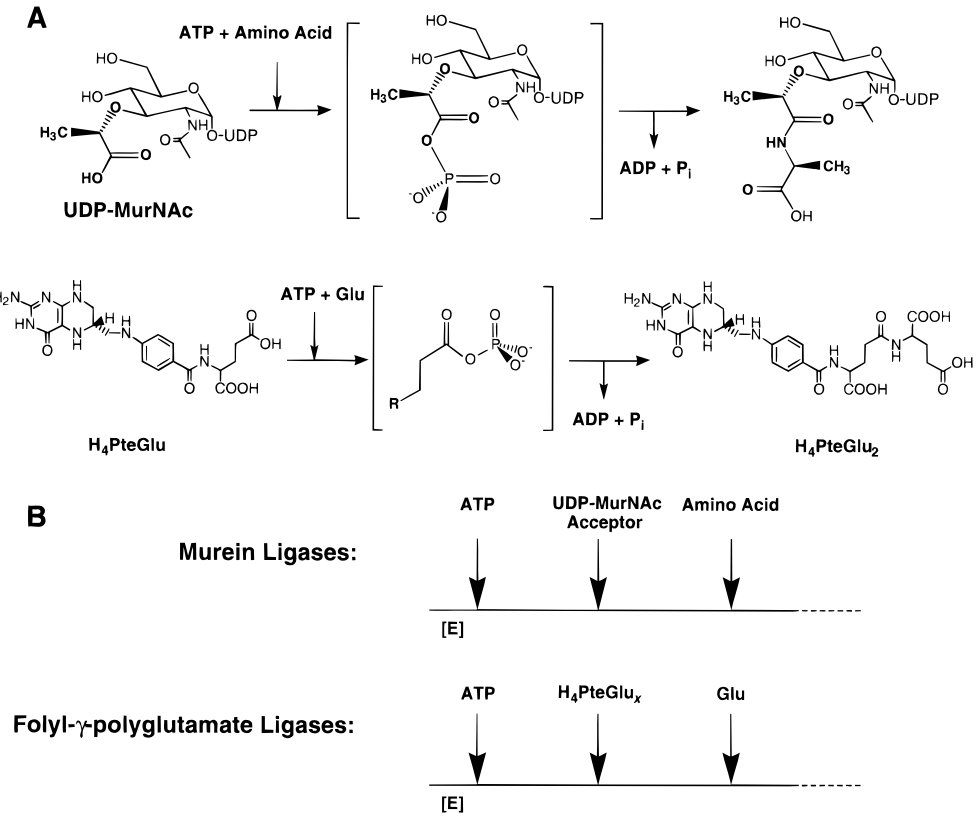

FIGURE 2: Murein ligases MurC, -D, -E, and -F share parallel enzymology with the folyl-γ-polyglutamate ligases. Panel A: Each of these four murein proteins catalyzes the ligation of different amino acids into a growing peptide chain from UDP-MurNAc via an acyl phosphate intermediate. Evidence for such an intermediate has been presented in the case of MurC (the reaction shown) and MurD (Falk et al., 1996; Tanner et al., 1996). The folyl-γ-polyglutamate ligases catalyze a similar ligation of L-glutamate via the analogous acyl phosphate intermediate (Banerjee et al., 1988). Panel B: The MurF protein has been demonstrated to utilize a sequential kinetic mechanism for the forward reaction (Anderson et al., 1996). This forward kinetic mechanism has also been demonstrated with the folyl-γ-polyglutamate ligase from porcine liver (Cichowicz & Shane, 1987), *Lactobacillus casei* (Bognar & Shane, 1983), and *Corynebacterium* species (Shane, 1980). In the figure, **$H_4PteGlu_x$** represents 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, ***x*** indicates a variable number of glutamyl moieties, and **R** represents the remainder of H_4PteGlu.

in region II, especially with regard to the Asp-His-aliphatic-Asp motif (Table 2). However, a one-base shift in the reading frame led to a translation that encoded the region II conserved sequence (shown in Figure 1). It seems likely that a sequencing error has been made that has displaced the correct reading frame in this region. Region III further downstream is a short concentrated set of acidic residues that is conserved among most all the murein sequences in the form of a dyad.

These *mur* protein sequences were used to define a query profile according to the statistical method of Gribskov et al. (1987, 1989) in order to search the SWISSPROT data base. This search exposed other functionally related, non-murein enzymes (Figure 1). Folyl-γ-polyglutamate ligase catalyzes the ATP-dependent addition of successive glutamyl residues to either a folate monoglutamate in eukaryotes or to a dihydropteroate in prokaryotes (Figure 2). Both prokaryotic and eukaryotic forms of the enzyme harbor the signature regions I and II and elements of region IV. The acidic amino acid residues of murein ligase region III are present but appear somewhat more diffuse in the folyl-γ-polyglutamate ligases.

In addition to these ligases, all four homologous regions were identified in a protein encoded by the *capB* gene of *Bacillus anthracus* (Figure 1) (Makino, 1989). *CapB* is part of a three gene cluster that is implicated in the synthesis of capsule (poly-γ-D-glutamate) in this organism (Makino, 1989). A function for *capB* has not been demonstrated biochemically. On the basis of this analysis, we would predict that it encodes a ligase that polymerizes D-glutamyl residues.

Table 2: Potential Frameshift in Rhizobial murD Sequence L25875

sequence source	frame	encoded sequence[a]
Lineup consensus		LNV teDHMDR -12X- K
L25875	1)	LNL RRSPGSP -12X- R
1 bp frameshift[b]	2)	LNL xxDHLDR -12X- R
2 bp frameshift	3)	LNL xxITWIA -12X- S

[a] Residues underlined are heavily conserved in Figure1. [b] Frameshift of L25875, postulated within the 6 bp region encoding the amino acids marked x.

Production and Assay of Mutant Alleles in Conserved Region II Residues of the E. coli D-*Ala*-D-*Ala-Adding Enzyme*. This sequence alignment not only defines regions of homology but also identifies specific amino acids that are strongly conserved and therefore predicted to be important to either folding or function in this enzyme family. In order to test whether these residues, and hence the alignments in general, were of significance, we undertook several site-directed mutagenesis experiments in the conserved glutamate and histidine residues of region II (Figure 1) using the *E. coli* D-Ala-D-Ala-adding enzyme as a model. Previous examination of this protein in the setting of a fusion with glutathione

Table 3: Point Mutants in Conserved Glu and His Residues of Region II

E158 mutants			H188 mutants		
mutant[a]	relative activity	fold reduction	mutant	relative activity	fold reduction
wild type	100		wild type	100	
E158A	0.0221	4520	H188A	0.493	203
E158G[b]	<0.222	>451	H188G	0.467	214
E158D	0.406	246	H188N	0.0539	1860
			H188D	0.0334	2990

[a] Both wild-type and mutant enzymes are purified glutathione S-transferase fusions. [b] Activity estimate limited due to reduced solubility of this mutant.

S-transferase (GST::MurF) has shown that its kinetic parameters are identical to those of unfused and purified recombinant protein (Anderson et al., 1996). Therefore, site-directed mutagenesis experiments performed in the setting of this GST::MurF fusion should allow for expression of each mutant with a benign tag that offers a convenient means of purification from the endogenous native form. All mutants so created were purifiable in this manner.

Thus, we systematically replaced glutamate 158 of the *E. coli* MurF protein with alanine, glycine, or the conservative isostere aspartate and measured residual enzymatic activity at concentrations of substrate that are saturating for the wild type (Table 3). All changes strongly reduced catalytic turnover. Interestingly, though 246-fold reduced in specific activity relative to the wild-type, the E158D mutation retains an 18-fold increase in activity over the corresponding aliphatic replacement E158A. This would be consistent with a mispositioned carboxylate in the E158D mutant that may still aid catalysis (or folding) in a manner not available to the E158A mutant.

Encouraged by the results with glutamate, we similarly mutated histidine 188 to alanine, glycine, the conservative isostere asparagine, and aspartate (Table 3), the only amino acid other than histidine to appear at this position in the alignment (in Fpg_Sce). As with the glutamate mutations, all four alterations were strongly damaging to catalytic turnover. However, it was the conservative isostere H188N that was now 9-fold further reduced in activity relative to the aliphatic replacements. Introduction of a negative charge at this position (H188D) only exacerbated this inactivation. One explanation for these results could be the loss of a conjugate acid or base (histidine 188) that assists in catalytic function. In the aliphatic mutants, a water molecule could then act as a poor replacement for the missing imidazole, whereas in H188N, an asparagine would occupy this space unproductively. However, all efforts to enhance catalytic turnover in the H188A or H188G mutant enzymes in vitro by complementation with widely varied concentrations of imidazole failed.

Sequencing of Conditional Mutations in Murein Ligases Reveals Clustering in Region IV. In order to gain additional support for the importance of any of these regions to cell viability, we investigated several *E. coli* strains that harbor temperature-conditional alleles of the *mur* genes. Despite significant phenotypic data, the specific defects in these mutants have not been elucidated. Several representative mutants, ST222 (*murC3*), TKL11 (*murE1*), and TKL46 (*murF2*) were obtained from the *E. coli* Genetic Stock Center. From each defective strain, the mutant *mur* allele was cloned by PCR using Pfu polymerase. The defective allele and surrounding DNA from each strain was recovered in two parallel PCR reactions and sequenced. In each case, the sequence of the separate isolates agreed exactly. Interestingly, each of the deviations from the wild-type sequence, tabulated in Table 4, occur either in or just after region IV.

Table 4: Defects harbored by murein mutants of *Escherichia coli*

strain[a]	allele[b]	base change[c]	amino acid change
ST222	murC3	$G^{1030}A$	$Gly^{344}Asp$
TKL11	murE1	$G^{1029}A$	$Glu^{344}Lys$
		$G^{1482}T$	$Ala^{495}Ser$
TKL46	murF2	$G^{860}A$	$Ala^{288}Thr$
		$G^{1323}A$	silent

[a] Strains obtained from the *E. coli* Genetic Stock Center. [b] Accession numbers for mutant sequences: *murC3*, U67892; *murE1*, U67894; *murF2*, U67893. [c] Base pairs altered from start of reading frame.

Table 5: Residual Activity of the MurF2 Protein As Compared to the Wild Type

protein	genotype	specific activity (μmol min^{-1} mg^{-1})	relative activity
	Assayed at 30 °C		
GST::MurF	$murF^+$	11.2	1
GST::MurF2	murF2	0.062	0.0055
	Assayed at 42 °C		
GST::MurF	$murF^+$	13.9	1
GST::MurF2	murF2	0.040	0.0028

The mutant form of the D-Ala-D-Ala-adding enzyme (*murF2*) from TKL46 was expressed as a GST fusion in order to test whether this allelic variant had impaired catalytic activity in vitro. The mutant protein, GST::MurF2, was purified by affinity chromatography on glutathione−agarose. GST::MurF protein of wild-type sequence was prepared similarly (Anderson et al., 1996). The purified GST::MurF2 fusion protein was assayed for catalytic activity in parallel with the wild-type GST::MurF fusion. At 30 °C, the mutant was 181-fold reduced in ligase specific activity compared to the wild-type enzyme (Table 5). Residual ligase activity of the GST::MurF2 protein was further reduced when assayed at 42 °C, resulting in an overall 360-fold reduction in specific activity. Apparently, the temperature-sensitive mutant bacterium can ill afford this further 2-fold diminution of activity induced by temperature shift; the meager residual D-Ala-D-Ala-adding enzyme activity falls below the threshold activity required for viable cell wall synthesis.

DISCUSSION

The results of the alignment in Figure 1 indicate that the MurC, -D, -E, and -F proteins, murein peptide ligases, and the folyl-γ-polyglutamate ligases share four highly conserved regional features as well as several conserved amino acid residues. One obvious region is the nucleotide binding motif that seems certain to be involved in ATP binding. Several other conserved residues are intriguing, because their functions are far less clear. Among these are the invariant glutamate in region II as well as a contextually conserved histidine. One test of the veracity of this alignment was the discovery of this conserved region encoded in a Rhizobial *murD* sequence when translated via a single base pair frameshift. Further tests of the alignment were sought through site-directed mutagenesis of these residues in a setting in which the mutant protein could be easily isolated

from wild-type activity and examined for catalytic activity. Mutation of Glu158 and His188 in UDP-MurNAc-tripeptide: D-Ala-D-Ala-adding enzyme resulted in uniformly damaging effects to catalytic turnover, validating these residues as important for either catalysis or proper folding. A single exception to the conserved histidine is found in the *Saccharomyces* folyl-γ-polyglutamate ligase, which bears aspartate in place of histidine at this locus. Yet aspartate was not an acceptable replacement for histidine in the *E. coli* D-Ala-D-Ala-adding enzyme. If it is truly found in the *Saccharomyces* folyl-γ-polyglutamate ligase, then either accommodating changes in other residue(s) are present that allow function or the aspartate interacts with the prefolyl substrate in a manner that is not applicable to the UDP-MurNAc-tripeptide.

Further evidence correlating regions identified through the sequence alignments and effects on structure or function were obtained through the sequencing of several alleles of the murein ligases harbored in a set of temperature-conditional lethal *E. coli* mutants. Single point mutations were identified in the *murC3*, *murE1*, and *murF2* alleles that reside largely in or near region IV. Expression of the mutant *murF2* allele confirmed both the lowered catalytic efficiency and the temperature sensitivity of this allelic D-Ala-D-Ala-adding enzyme. We cannot conclude whether these defects result in protein structural deformations or a combination of other perturbations. However, these mutants suggest that region IV may tolerate only limited sequence variations; these results tie small variations in this region to effects in vivo.

The substrate specificities of the bacterial and mammalian folyl-γ-polyglutamate ligases differ substantially (Cichowicz & Shane, 1987) despite high sequence homology. This seems most consistent with a model in which the conserved residues of this alignment are not involved directly in determining specificity for either the amino acid or carboxylate substrates. Although as a group the Mur proteins bear specific homologies in regions I−IV, each protein catalyzes the ligation of a distinct amino acid to the growing stem peptide. It seems reasonable that the residues directing this specificity should be conserved within any single type of Mur enzyme. In this regard, the C-terminal portions of each Mur protein beyond region IV show little global homology yet are relatively undiverse within any specific enzyme. Perhaps the individuality of each ligase with respect to amino acid specificity is encoded here.

Recently, affinity labeling experiments have shown that a specific cysteine (C414 of the *E. coli* protein) of MurD can be protected from derivatization by substrate (Vaganay et al., 1996). However, this cysteine is found only in MurD sequences and has no apparent direct homologs (data not shown). Although it may be involved in substrate binding in this enzyme, it is unlikely to be generally involved in catalysis.

Beyond these homologies, several further parallels between these enzyme families can be drawn as shown in Figure 2. Both enzymes catalyze the ATP-dependent formation of an amide bond with production of ADP and inorganic phosphate. The kinetic mechanism for the folyl-γ-polyglutamate ligase from porcine liver (Cichowicz & Shane, 1987), *Lactobacillus casei* (Bognar & Shane, 1983), and *Corynebacterium* species (Shane, 1980) has been determined. Each of these enzymes proceeds in the forward direction via an ordered ternary mechanism, a kinetic route also utilized in the case of the *murF*-encoded D-Ala-D-Ala ligase (Anderson et al., 1996). Further, the reaction catalyzed by the MurC protein proceeds via an acyl phosphate intermediate (Falk et al., 1996). Similar studies with the MurD ligase are consistent with such an intermediate during D-glutamate addition (Vanganay et al., 1996). Indeed, phosphinate-containing transition-state mimetics are micromolar inhibitors of this enzyme (Tanner et al., 1996). Correspondingly, both bacterial and mammalian forms of the folyl-γ-polyglutamate ligases catalyze amide bond formation through the analogous acyl phosphate intermediate (Figure 2) (Banerjee et al., 1988). Taken together, these mechanistic similarities as well as the highly conserved nature of regions of sequence critical to proper catalytic function argue that these proteins are part of a larger superfamily.

By comparison, the *ddlB* gene encodes a ligase that converts two molecules of D-alanine to the MurF dipeptide substrate D-Ala-D-Ala (Zawadzke et al., 1991). This enzyme utilizes ATP, possesses the same ordered forward kinetic mechanism, and functions via an acyl phosphate intermediate (Figure 2) (Mullins et al., 1990). However, despite these functional homologies, this ligase does not match the protein homology pattern of the four Mur enzymes. In this light, the finding that the folyl-γ-polyglutamate ligases possess partially or wholly conserved structural features that are similarly localized was unanticipated. This suggests that several protein structural solutions are available that lead to a functionally similar ligase. Indeed, the X-ray crystallographic structure of the DdlB ligase shares strong three-dimensional homologies with that of glutathione synthetase (Fan et al., 1995), though no regions of primary structural identity are readily apparent. Neither of these latter proteins fits the pattern of Figure 1, and therefore they are not members of the murein ligase superfamily. But they do appear to represent a separate ligase tertiary structural family that performs similar enzymatic transformations in an analogous fashion.

None of the protein structures in Figure 1 has been solved by either NMR or X-ray crystallography. However, the three-dimensional structure of any one of these enzymes may allow a credible model to be built for any of the others, at least with respect to the active site. The structural and functional homologies of these Mur proteins open the possibility that a small-molecule inhibitor of more than one of these enzymes might be designed or discovered. Since each of these murein gene products is essential, such a multimodal inhibitor should be a formidable weapon against bacteria, with the added benefit of decreasing dramatically the likelihood of resistance being acquired through a single point mutation of the target.

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