Articles

Invariant Amino Acids in the Mur Peptide Synthetases of Bacterial Peptidoglycan Synthesis and Their Modification by Site-Directed Mutagenesis in the UDP-MurNAc:L-Alanine Ligase from *Escherichia coli*†

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ABSTRACT: The comparison of the amino acid sequences of 20 cytoplasmic peptidoglycan synthetases (MurC, MurD, MurE, MurF, and Mpl) from various bacterial organisms has allowed us to detect common invariants: seven amino acids and the ATP-binding consensus sequence GXXGKT/S all at the same position in the alignment. The Mur synthetases thus appeared as a well-defined class of closely functionally related proteins. The conservation of a constant backbone length between certain invariants suggested common structural motifs. Among the other enzymes catalyzing a peptide bond formation driven by ATP hydrolysis to ADP and P_i, only folylpoly-γ-L-glutamate synthetases presented the same common conserved amino acid residues, except for the most N-terminal invariant D50. Site-directed mutageneses were carried out to replace the K130, E174, H199, N293, N296, R327, and D351 residues by alanine in the MurC protein from *Escherichia coli* taken as model. For this purpose, plasmid pAM1005 was used as template, MurC being highly overproduced in this genetic setting. Analysis of the V_{max} values of the mutated proteins suggested that residues K130, E174, and D351 are essential for the catalytic process whereas residues H199, N293, N296, and R327 were not. Mutations K130A, H199A, N293A, N296A, and R327A led to important variations of the K_m values for one or more substrates, thereby indicating that these residues are involved in the structure of the active site and suggesting that the binding order of the substrates could be ATP, UDP-MurNAc, and alanine. The various mutated *murC* plasmids were tested for their effects on the growth, cell morphology, and peptidoglycan cell content of a *murC* thermosensitive strain at 42 °C. The observed effects (complementation, altered morphology, and reduced peptidoglycan content) paralleled more or less the decreased values of the MurC activity of each mutant.

The biosynthesis of bacterial peptidoglycan is a complex two-stage process (van Heijenoort, 1995, 1996). The first stage involves the assembly of the disaccharide peptide monomer unit by enzymes located in the cytoplasm or at the inner surface of the cytoplasmic membrane, whereas the second stage concerns the polymerization and maturation reactions that take place at the outer surface of the cytoplasmic membrane. The peptide moiety of the monomer unit is assembled stepwise by the successive addition of L-alanine, D-glutamate, a diaminoacid (diaminopimelate or L-lysine), and a dipeptide (generally D-Ala-D-Ala) to UDP-*N*-acetylmuramate. Each step is catalyzed by a specific synthetase which has no cross-activity with the other steps. These peptide synthetases are designated as MurC, MurD, MurE, and MurF, respectively, and they all bring about nonribosomal peptide bond formation with concomitant hydrolysis of ATP to ADP and P_i. They most likely operate by an essentially similar mechanism which entails carboxyl activation of the nucleotide substrate to an acylphosphate intermediate followed by nucleophilic attack by the amino group of the condensing amino acid or dipeptide, with elimination of phosphate and subsequent peptide bond formation. The existence of acylphosphate and tetrahedral intermediates has been proposed with the L-alanine- and D-glutamate-adding enzymes (Falk *et al*., 1996; Liger *et al*., 1996; Tanner *et al*., 1996; Vaganay *et al*., 1996). The genes (*murC, murD, murE, and murF*) for these synthetases have been identified, cloned, and sequenced in *Escherichia coli*, *Haemophilus influenzae*, and *Bacillus subtilis*. Recently, the *mpl* gene for the synthetase catalyzing the formation of UDP-MurNAc-tripeptide by addition of L-Ala-γ-D-Glu-*meso*-A_2pm onto UDP-MurNAc in the recycling process of peptidoglycan was identified in *E. coli* and *H. influenzae* (Mengin-Lecreulx *et al*., 1996).

Presently, the therapeutic value of the antibiotics in clinical use is largely reduced or totally annihilated by the emergence and spreading of various resistance mechanisms among bacterial pathogens. To tackle this problem the definition of targets susceptible to inhibition has become a high priority task for the search of novel antibacterials. Owing to their high specificity, their uniqueness, and their occurrence only in eubacteria, the synthetases responsible for the assembly

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Table 1: Mutations Generated in the *murC* Gene Product

plasmid	mutation[a]	oligonucleotide[b]	new restriction sites[c]
pABCK130A	K130A	5′-CCGGAACGCACGGC**GCC**ACGACAACCACCG-3′	*Hae*II, *Nar*I
pABCE174A	E174A	5′-GGTACCTGATTGCCG**CT**GCAGATGAGAGTG-3′	*Pst*I
pABCH199A	H199A	5′-ATATCGAAGCCGAC**GC**CATGGATACCTACC-3′	*Nco*I, *Sty*I
pABCN293A	N293A	5′-CGCCAGGTCGTCAT**GCG**GCGCTGAACGCCG-3′	*Hae*II
pABCN296A	N296A	5′-CGTCATAACGCGCT**AGC**CGCCGCAGCTGCG-3′	*Bfa*I
pABCR327A	R327A	5′-GGGGACTGGTCG**GGCC**TTTGATTTCCTCGG-3′	*Sau*96I
pABCD351A	D351A	5′-GCAATGCTGGTCGA**C**G**C**CTACGGCCACCAC-3′	*Hinc*II, *Sal*I

[a] Amino acids are represented by their one-letter abbreviation, and the number indicates the localization of mutated residue in the amino acid sequence of MurC. [b] Mutations of the *murC* gene sequence that have been introduced in the oligonucleotides are indicated in bold. [c] In order to facilitate the final selection or confirmation of mutated plasmids, mutations that in addition generate new restriction sites in the gene sequence were chosen.

of the peptide moiety of peptidoglycan are potential targets of particular interest. As yet, no information on the amino acid residues involved in the active site of these enzymes is available. Homologies between the amino acid sequences of the MurC, MurD, MurE, and MurF synthetases from *E. coli* have been elicited, suggesting that their genes had evolved from a common ancestral gene (Ikeda *et al*., 1990). In the present paper, the comparison of the amino acid sequences of 20 Mur synthetases from various bacterial organisms has allowed us to detect common amino acid invariants. Since it is essential to determine whether such amino acid residues are in someway involved in the catalytic mechanism, site-directed mutageneses of these invariants were made with the L-alanine-adding enzyme (MurC) from *E. coli*. The various mutants were analyzed in terms of complementation, enzymatic parameters, and peptidoglycan synthesis.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. *E. coli* strains DH5α (*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Φ80 d*lacZ* ΔM15) (Bethesda Research Laboratories) and PC2453 (*purE murC*[Ts] *phx lam*[*lam*] *rpsL*) (Phabagen collection, Department of Molecular Cell Biology, State University of Utrecht, Utrecht, The Netherlands) were used as hosts for plasmids as well as for the overproduction of wild-type and mutant MurC proteins. Strain UMAS83, which is blocked in the expression of the *murC* gene at the restrictive temperature of 42 °C, was kindly provided by E. Realo and M. T. Bocquel (unpublished data). This strain, which carries an inactivated copy of *murC* in the chromosome and an intact copy of *murC* on a plasmid whose replication is thermosensitive, was used for the screening of plasmids expressing a functional *murC* gene product. *E. coli* strain BMH71−18 *mutS* defective in mismatch repair was used in site-directed mutagenesis experiments (Deng & Nickoloff, 1992). Plasmid pAM1005 carrying the *murC* gene expressed under the control of the strong *trc* promoter in p*Trc*99A vector has been previously described (Liger *et al*., 1995). 2YT (Miller, 1972) was used as a rich medium for growing cells, and growth was monitored by measuring the culture absorbance at 600 nm. For strains carrying drug resistance genes, antibiotics were used at the following concentrations: ampicillin (100 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}), and tetracycline (15 μg mL^{-1}).

General DNA Techniques and E. coli Cell Transformation. Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (Sambrook *et al*., 1989). Standard procedures for endonuclease digestion, ligation, and agarose electrophoresis were used (Davis *et al*., 1972; Sambrook *et al*., 1989). DNA sequencing was performed according to Sanger *et al*. (1977), using the T7 sequencing kit from Pharmacia. *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (1979) or by electroporation.

Site-Directed Mutagenesis. Site-directed mutagenesis of the *E. coli* MurC enzyme was performed by using the Transformer site-directed mutagenesis kit purchased from Clontech (Palo Alto, CA) based on the recently developed site-directed mutagenesis method of Deng and Nickoloff (1992). This method works by simultaneous annealing of two oligonucleotide primers to one strand of a denaturated plasmid; one primer introduces the desired mutation in the gene and the other primer mutates a unique restriction site in the plasmid vector sequence for the purpose of selection. The latter primer which is common to all mutagenesis experiments (5′-TTGGTGCGGACATCTCGGTAG-3′) was defined for suppression of the unique *Eco*RV site lying within the *lacI*^{q} gene of the target plasmid pAM1005 (with no change in the aminoacid sequence of LacI). Table 1 shows the sequences of the oligonucleotides used for introduction of specific mutations within the *murC* gene. In each case, after annealing of both primers, standard DNA elongation, and ligation steps, the resulting mixture of mutated and wild-type plasmids was transformed into the *E. coli mutS* strain BMH71-18 defective in mismatch repair. Transformants selected in 2YT-ampicillin medium were pooled (more than 10^3), and their plasmid DNA was prepared and digested by *Eco*RV (to eliminate wild-type plasmids carrying an intact *Eco*RV site). This reaction mixture was then used to transform strain DH5α. As predicted, DNA sequencing of plasmids resistant to *Eco*RV digestion showed that most of them also carried the expected mutation in the *murC* gene. In each case, one of the mutated clones was chosen and used for further investigations.

Preparation of Crude Enzyme. PC2453 cells carrying either the pAM1005 (wild-type *murC*) or one of the pAB plasmids indicated in Table 1 (mutated *murC*) were grown exponentially at 30 °C in 2YT-ampicillin medium (500 mL cultures). When the optical density of the culture reached 0.2, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and growth was continued for 5 h. Cells were harvested in the cold and washed with cold 0.02 M potassium phosphate buffer, pH 7.2, containing 2.5 mM β-mercaptoethanol and 1 mM EDTA (buffer A). The wet cell pellet was suspended in 5 mL of the same buffer and sonicated in the cold until disruption of all cells. The resulting suspension was centrifuged at 4 °C for 20 min at

200000*g* in a Beckman TL100 centrifuge and pellets discarded. SDS−PAGE analysis of proteins from these crude extracts was performed as previously described (Laemmli & Favre, 1973), using 12% polyacrylamide gels. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Standard Assay for UDP-MurNAc:L-Alanine Ligase Activity. The L-alanine-adding activity was routinely assayed according to Liger *et al*. (1995), by following the formation of UDP-MurNAc-L-[^{14}C]Ala in a mixture containing, in a final volume of 50 μL, 0.1 M Tris-HCl buffer, pH 7.8, 20 mM $MgCl_2$, 20 mM ammonium sulfate, 1 mM L-1-aminoethylphosphonic acid, 5 mM ATP, 0.5 mM UDP-MurNAc, 0.5 mM L-[^{14}C]alanine (2 kBq), and enzyme (25 μL of an appropriate dilution in buffer A). The mixture was incubated for 30 min at 37 °C and the reaction was stopped by addition of 10 μL of acetic acid. The product UDP-MurNAc-L-[^{14}C]-Ala was separated from L-[^{14}C]Ala by reverse-phase HPLC on a Nucleosil $5C_{18}$ column (4.6 × 150 mm; Alltech France, Templeuve, France) using 50 mM ammonium formate, pH 4.3, at a flow rate of 0.6 mL min^{-1}. Detection was performed with a radioactive flow detector (model LB506-C1, Berthold, Bad Wildbad, Germany) using the Quicksafe Flow 2 scintillator (Zinsser Analytic, Maidenhead, U.K.) at 0.6 mL min^{-1}. Quantification was carried out with a computer connected to the detector and equipped with a Berthold HPLC software.

Isolation of Sacculi and Quantitation of Peptidoglycan. Cells of UMAS83 carrying either the control vector p*Trc*99A, the pAM1005 plasmid (wild-type *murC*), or one of the pAB plasmids (mutated *murC*), indicated in Table 1, were grown exponentially at 30 °C in 2YT-ampicillin medium (500 mL cultures). At the appropriate cell concentration (3×10^6 mL^{-1}), the temperature of the culture was increased to 42 °C and incubation was continued for about 5 h, in which time the optical density of the culture of control UMAS83-(p*Trc*99A) cells reached a plateau value of about 0.7. Thereafter, cells were rapidly chilled to 0 °C, harvested in the cold, and washed with a cold 0.85% NaCl solution. Bacteria were then rapidly suspended under vigorous stirring in 40 mL of a hot (95−100 °C) aqueous 4% SDS solution for 30 min. After the suspensions set overnight at room temperature, they were centrifuged for 30 min at 200000*g* in a Beckman TL100 centrifuge and the pellets were washed several times with water. Final suspensions made in 5 mL of water were homogenized by brief sonication. Aliquots were hydrolyzed (6 M HCl, 95 °C, 16 h) and analyzed with a Biotronik amino acid analyzer (model LC-2000, Frankfurt, Germany) equipped with a column of DC-6A (Dionex, Sunnyvale, CA) and a Spectra-Glo fluorometer (Gilson, Villiers-le-Bel, France). *o*-Phthalaldehyde/β-mercaptoethanol was used as the postcolumn derivatization reagent. The peptidoglycan content of the sacculi was expressed in terms of its muramic acid content (Mengin-Lecreulx *et al*., 1982, 1985).

Complementation Test. Cells of the thermosensitive mutant strain UMAS83 were made competent as described above and transformed by the various plasmids to be tested. The cell suspension mixed with plasmid DNA was kept on ice for 4 h before heating for 3 min at 42 °C. Then, 400 μL of 2YT medium was added and cells were incubated at 30 °C for 2 h. Aliquots from the final suspensions were plated onto two 2YT-ampicillin plates, one incubated at 30 °C and the other at 42 °C. Growth was observed after 24 h of incubation.

RESULTS

Alignment of the Cytoplasmic Peptidoglycan Synthetases. The sequences of 20 cytoplasmic peptidoglycan synthetases (MurC, MurD, MurE, MurF, and Mpl) from Gram-negative (*E. coli, H. influenzae, Borrelia burgborferi, Cyanobacterium synechocystis, Porphyromonas gingivalis,* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*B. subtilis*) were aligned using the CLUSTAL W program (Thompson *et al.*, 1994), and the final alignment presented was optimized manually (Figure 1). For all these synthetases, seven amino acid residues and the ATP-binding consensus sequence GXXGKT/S are found at the same position in the alignment. Taking the L-alanine-adding enzyme of *E. coli* as a reference, these residues are aspartate 50 in the N-terminal part of the protein, glutamate 174, histidine 199, asparagines 293 and 296, arginine 327, and in the C-terminal part aspartate 351 (Figure 1). These sequences were also compared with those of other synthetases (D-Ala:D-Ala ligases, glutamine synthetases, γ-glutamylcysteine synthetases, glutathione synthetases, and folylpoly-γ-L-glutamate synthetases) which are available in data banks and which catalyze a similar amide or peptide bond-forming reaction. Only folylpoly-γ-L-glutamate synthetases (FolC) present the same common conserved aminoacid residues as the peptidoglycan synthetases except for the most N-terminal invariant D50. FolC is involved in the addition of L-glutamate residues onto the folic acid coenzyme. Furthermore, the size of the sequences between the invariant residues is of particular interest (Table 2). For instance, the number of amino acid residues between E174 and H199 varies from 26 in the L-alanine-adding enzymes, 27 and 28 in the D- and L-glutamate-adding enzymes, respectively, 29 in the diaminopimelate-adding enzymes, 31 in the dipeptide-adding enzymes, and 33 in the tripeptide-adding enzymes. It thus seems correlated with the size of the amino acid or peptide substrate. Moreover, the number of amino acids was very well conserved between N296 and R327, and only fairly well conserved between D50 and G126, H199 and N293, or R327 and D351.

Site-Directed Mutagenesis of the Invariant Residues. Site-directed mutageneses were carried out to replace the K130, E174, H199, N293, N296, R327, and D351 residues by alanine in the MurC protein taken as model. For this purpose, plasmid pAM1005 was used as template, the UDP-MurNAc:L-Ala ligase being highly overproduced in this genetic setting. All the mutations were confirmed by DNA sequencing. Crude extracts were prepared as previously described (Liger *et al.*, 1995) from the PC2453 strain harboring one or another of the mutated *murC* plasmids. Analysis by SDS−PAGE (Figure 2) revealed that the seven mutant MurC proteins were overproduced to the same high level as the wild-type MurC directed by the pAM1005 plasmid and were particularly stable in the host strain considered (no obvious proteolysis). In crude extracts, they accounted for more than 50% of the cytoplasmic proteins.

Enzymatic Properties of the MurC Mutants. The L-alanine-adding activity was measured under the conditions of the MurC standard assay at cell pH 7.8 in crude extracts from the PC2453 strain overproducing the wild-type or mutant proteins (Table 3). In all mutants, it was lower than

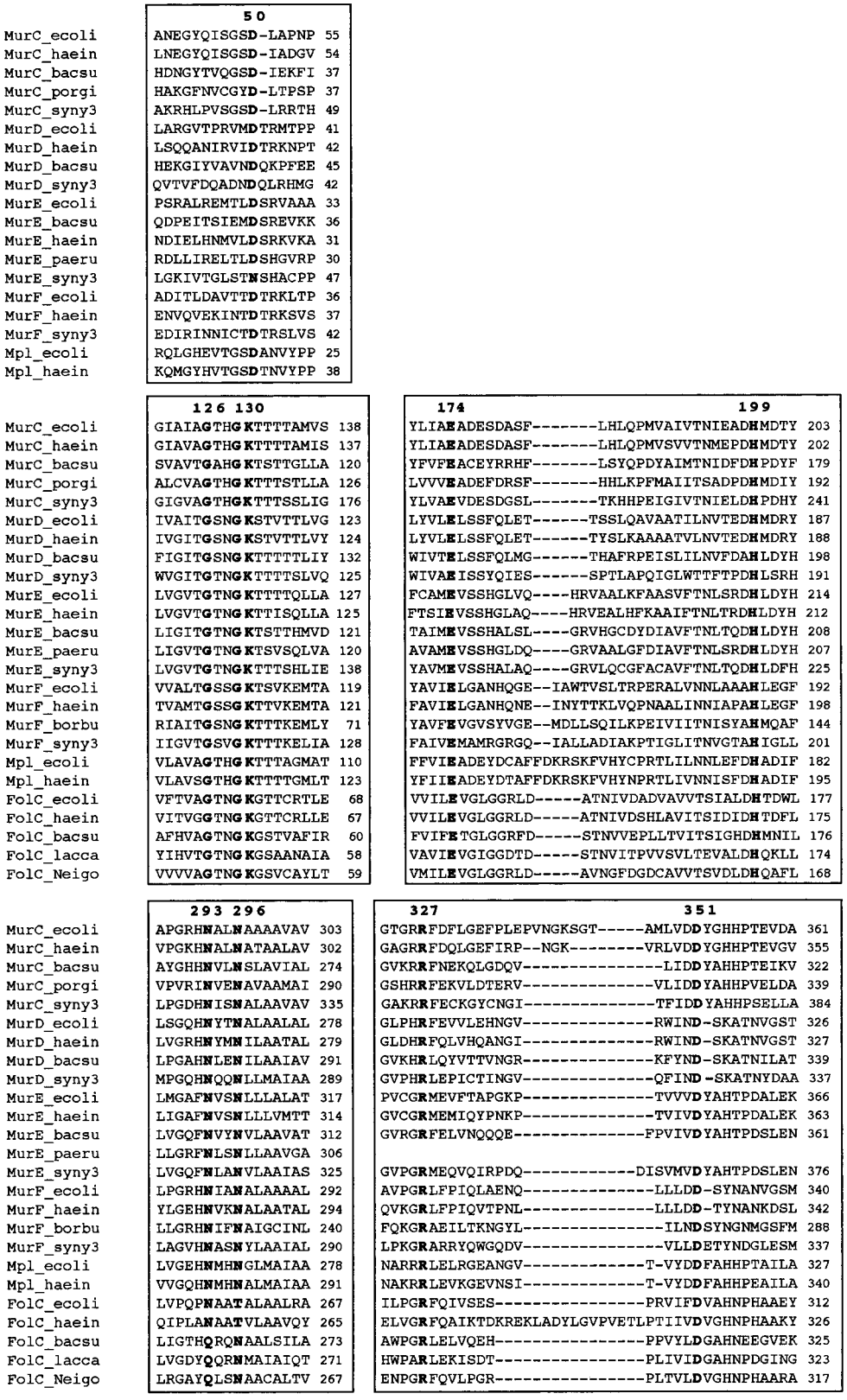

FIGURE 1: Multiple alignments of the amino acid sequences of the Mur and folylpoly-γ-L-glutamate synthetases (FolC) from *Escherichia coli* (ecoli), *Haemophilus influenzae* (haein), *Porphyromonas gingivalis* (porgi), *Bacillus subtilis* (bacsu), *Cyanobacterium synechocystis* (syny3), *Lactobacillus casei* (lacca), *Neisseria gonorrhoeae* (neigo), and a fragment of MurE and MurF from *Pseudomonas aeruginosa* (paeru) and *Borrelia burgdorferi* (borbu), respectively. Only clusters containing the invariant amino acid residues are shown. Conserved amino acids are printed in bold and their numbering refers to *E. coli* MurC.

in the wild-type strain, but important differences ranging from an only 60% decrease in N293A to an undetectable level in E174A were observed. In particular, mutants K130A and D351A showed a very low but yet detectable activity. The apparent K_m values for the ATP, L-alanine, and UDP-MurNAc substrates of the mutant and wild-type proteins were determined at pH 7.8 (Table 4). In each case, the mutation led to important variations of the K_m values for one or two of the substrates. In the R327A enzyme, a high 114-fold increase of the K_m for alanine was only observed. With the

Table 2: Number of Residues between the Invariant Amino Acids in Synthetases MurC, MurD, FolC, MurE, MurF, and Mpl

	D_{50}-G_{126}	K_{130}-E_{174}	E_{174}-H_{199}	H_{199}-N_{293}	N_{296}-R_{327}	R_{327}-D_{351}	amino acid or peptide substrate
MurC_ecoli	77	45	26	95	32	25	L-Ala
MurC_bacsu	77	39	26	90	32	15	L-Ala
MurC_haein	76	44	26	95	32	20	L-Ala
MurC_porgi	82	46	26	93	32	16	L-Ala
MurC_syny3	121	45	26	89	32	16	L-Ala
MurD_bacsu	82	45	27	88	32	16	D-Glu
MurD_ecoli	77	43	27	87	32	16	D-Glu
MurD_haein	77	43	27	87	32	16	D-Glu
MurD_syny3	78	45	27	93	32	16	D-Glu
FolC_ecoli		87	28	85	30	14	L-Glu
FolC_bacsu		94	28	95	34	14	L-Glu
FolC_haein		86	28	85	30	30	L-Glu
FolC_lacca		94	28	95	34	14	L-Glu
FolC_neigo		87	28	97	32	14	L-Glu
MurE_ecoli	83	64	29	98	32	16	*meso*-A_2pm
MurE_bacsu	80	64	29	99	32	16	*meso*-A_2pm
MurE_haein	89	64	29	98	32	16	*meso*-A_2pm
MurE_paeru	82	64	29	94			*meso*-A_2pm
MurE_syny3	83	64	29	95	32	18	*meso*-A_2pm
MurF_ecoli	78	48	31	95	32	16	D-Ala-D-Ala
MurF_haein	79	49	31	91	32	16	D-Ala-D-Ala
MurF_borbu	>69	48	31	91	32	15	D-Ala-D-Ala
MurF_syny3	81	51	31	84	31	15	D-Ala-D-Ala
Mpl_ecoli	80	45	33	91	32	16	L-Ala-γ-D-Glu-*meso*-A_2pm
Mpl_haein	80	45	33	91	32	16	L-Ala-γ-D-Glu-*meso*-A_2pm

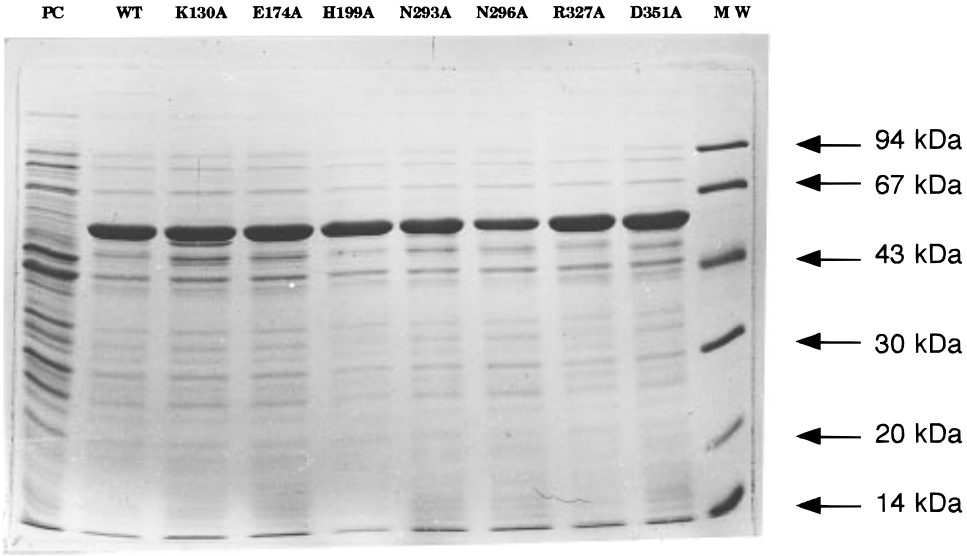

FIGURE 2: Overproducing of wild-type and mutant MurC proteins. Crude extracts from strains carrying plasmids with the wild-type or mutated *murC* gene (induced by IPTG) were analyzed by SDS−PAGE. Lanes: PC, WT, K130A, E174A, H199A, N293A, N296A, R327A, and D351A correspond to soluble fractions from PC2453, PC2453(pAM1005), PC2453(pABCK130A), PC2453(pABCE174A), PC2453-(pABCH199A), PC2453(pABCN293A), PC2453(pABCN296A), PC2453(pABCR327A), and PC2453(pABCD351A) cells, respectively. MW: molecular mass standards in kDa.

K130A and N296A enzymes, essentially the K_m values for ATP and UDP-MurNAc were greatly altered. To a much lesser extent, the same was true for the N293A enzyme. Finally, in the H199A enzyme, the K_m values for UDP-MurNAc and alanine were altered, whereas the K_m for ATP was not.

In vivo Activity of the Mutant MurC Proteins. The various mutated *murC* plasmids as well as the p*Trc*99A and wild-type pAM1005 control plasmids were tested for their ability to restore growth of thermosensitive strain UMAS83 at 42 °C. Moreover, after a shift at 42 °C in liquid medium, the cell morphology of the transformants was examined by optical microscopy and their peptidoglycan cell contents were determined (Table 5). The various mutants fell into three groups. Mutants H199A, N293A, and N296A grew well at 42 °C, presented a normal rod shape cell morphology, and possessed the same or slightly reduced (H199A) level of peptidoglycan as in the wild-type strain. On the other hand, mutants E174A, R327A, and D351A did not grow at 42 °C, presented an altered ovoid morphology, and possessed a reduced peptidoglycan content (58−61% of the wild type) as did the UMAS83(p*Trc*99A) control strain which had lost its *murC* gene at 42 °C. Finally, mutant K130A was in an intermediate situation since it presented a limited complementation with very small colonies at 42 °C and a slightly higher (66% of the wild type) peptidoglycan content. Except for R327A, the observed cell effects (complementation, morphology, and peptidoglycan content) paralleled more or less the decreasing values of the L-alanine-adding activity of each mutant.

Table 3: L-Alanine-Adding Activity of the MurC Mutant Proteins[a]

MurC protein	activity (nmol min^{-1} mg^{-1})	relative activity(%)
WT	1500	100
N293A	600	40
N296A	180	12
H199A	90	6
R327A	35.7	2.5
K130A	1.35	0.09
D351A	0.15	0.01
E174A	<0.01	

[a] The L-alanine-adding activity was determined under the standard conditions of the MurC assay described in Materials and Methods with crude extracts from IPTG-induced PC2453 cells, carrying either plasmid pAM1005 (wild-type *murC*) or one of the plasmids with mutated *murC*.

DISCUSSION

The enzyme-catalyzed peptide bond formation driven by ATP hydrolysis to ADP and P_i is not a frequently encountered biochemical reaction. Aside from the Mur synthetases considered here, which very specifically catalyze by this type of reaction the assembly of the peptide moiety of the cytoplasmic peptidoglycan precursors, only a few other enzymes with a similar catalytic mechanism have been found and extensively studied. However, neither glutamine synthetases, glutathione synthetases, nor D-alanine:D-alanine ligases have any amino acid sequence homology with the Mur synthetases. With their common ATP-binding site and seven invariant amino acid residues, the Mur synthetases appear as a well-defined class of closely functionally related proteins originating presumably from a common ancestor. It will be interesting to see whether the Mur synthetase sequences from eubacteria other than the few considered here will fit or not into this class. The conservation of a constant backbone length between certain invariants could suggest common structural motifs in these Mur synthetases. It must be stressed that these enzymes are highly specific in the sense that the substrates for one type of synthetase are not recognized by another type. For instance, the MurC and Mpl synthetases differ only by their amino acid (alanine) or tripeptide (L-Ala-γ-D-Glu-*meso*-A_2pm) substrate. Yet no cross-activities were observed (Liger *et al.*, 1995; Mengin-Lecreulx *et al.*, 1996). Mur synthetases must therefore possess very well-defined sites for the peptide moiety of the UDP-MurNAc-peptide and for the amino acid or peptide substrate. Perhaps the variation of the size of the H199-N293 segment with the size of the amino acid or peptide substrate is in someway involved with the highly specific substrate recognition. Future analysis of the 3D structure of Mur synthetases should enable to explain such strict specificities. The relatedness of the folylpolyglutamate synthetases with the Mur synthetases is at first sight unexpected considering the not too obvious structural analogies between tetrahydrofolate and UDP-MurNAc. Interestingly, the FolC synthetases with the same invariants are also encountered in eucaryotes such as yeast, mouse, and man.

The replacement of the invariant amino acids by alanine residues was undertaken by site-directed mutagenesis in MurC to determine how essential they were for the enzyme activity and consequently for cell viability. The high V_{max} values of MurC proteins H199A, N293A, N296A, and R327A clearly suggested that the mutated amino acids were not essential for the catalytic process, and presumably these mutations did not lead to important structural modifications of the protein. On the contrary, the very low V_{max} values or absence of activity in MurC K130A, E174A, and D351A indicated that the mutated amino acids were essential. Lysine 130 belongs to a well-known consensus ATP-binding site (Walker *et al.*, 1982). The K_m value of the mutant K130A for ATP is increased 25-fold, suggesting that in MurC this residue is involved in the interaction with ATP as the other proteins carrying this motif. In the case of the D-alanine:D-alanine ligase, two lysine residues seem to be implicated in the interaction with the phosphate groups of ATP (Shi &

Table 4: Kinetic Parameters of Wild-Type and Mutant MurC Proteins at Physiological pH 7.8[a]

protein	V_{max} (nmol min^{-1} [mg of protein]$^{-1}$)	K_m^{ATP} (mM)	K_m^{UM} (mM)	K_m^{Ala} (mM)	V_{max}/K_m^{ATP} (nmol min^{-1} mM^{-1} [mg of protein]$^{-1}$)	V_{max}/K_m^{UM} (nmol min^{-1} mM^{-1} [mg of protein]$^{-1}$)	V_{max}/K_m^{Ala} (nmol min^{-1} mM^{-1} [mg of protein]$^{-1}$)
WT	3264	0.10	0.09	0.11	32640 (100)	36267 (100)	29673 (100)
K130A	260	2.80	4.80		93 (0.28)	54 (0.15)	
H199A	1552	0.15	1.88	0.85	10347 (31.7)	826 (2.3)	1826 (6.2)
N293A	1946	0.54	0.50	0.19	3603 (11)	3892 (10.7)	10242 (34.5)
N296A	1506	7.02	2.55	0.50	215 (0.66)	591 (1.63)	3012 (10.2)
R327A	2372	0.27	0.30	12.6	8785 (27)	7907 (21.8)	188 (0.63)

[a] The numbers in parentheses are relative values, the enzymatic activity of the wild-type protein being considered as 100%. UM: UDP-MurNAc

Table 5: *In Vivo* Activity of Mutated MurC Enzymes

strain	mutation	growth on plates at 42 °C	cell morphology in liquid medium after growth at 42 °C[a]	peptidoglycan content[a,b] [nmol g of cell dry weight^{-1}]
UMAS83(pAM1005)		yes	normal rod shape	11 600 (100%)
UMAS83(pABCN293A)	N293A	yes	normal rod shape	11 900 (103)
UMAS83(pABCN296A)	N296A	yes	normal rod shape	11 300 (97)
UMAS83(pABCH199A)	H199A	yes	normal rod shape	9000 (78)
UMAS83(pABCK130A)	K130A	yes (slowly)	ovoid	7700 (66)
UMAS83(pABCE174A)	E174A	no	ovoid	7100 (61)
UMAS83(pABCR327A)	R327A	no	ovoid	6700 (58)
UMAS83(pABCD351A)	D351A	no	ovoid	6700 (58)
UMAS83(p*Trc*99A)		no	ovoid	6700 (58)

[a] Cells were grown exponentially in 2YT-ampicillin medium, first at 30 °C up to 3×10^6 cells $\times$ mL^{-1} and then for 5 h at 42 °C, the time at which the growth rate of the mutant strain UMAS83 begins to decrease (see Materials and Methods). [b] The peptidoglycan content of sacculi was expressed in terms of its muramic acid content.

Walsh, 1995). Residue E174, which appears as essential for the MurC activity, could belong to the second ATP-binding motif defined by Walker *et al*. (1982) and also described in FolC (Bognar *et al.*, 1987; Fusseneger and Meyer, 1996). In various enzymes, the acid residue of this second motif generally contributes to the chelation of the ATP−Mg^{2+} complexes (Walker *et al*., 1982; Glaser *et al*., 1991; Story & Steitz, 1992; Mitchell & Oliver, 1993). Such a glutamate residue has been located at position 270 in D-alanine:D-alanine ligase DdlB, and its mutation led to a great loss of activity (Shi and Walsh, 1995). Further experiments are required to ascertain this point.

Mutations K130A, H199A, N293A, N296A, and R327A led to more or less important variations of the K_m values for one or more substrates, thereby indicating that these invariant amino acids are involved in the structure of the active site. The MurC R327A protein is characterized by a 114-fold increase of the K_m for alanine whereas the K_m values for ATP and UDP-MurNAc are practically unchanged. This strongly suggested that R327 is involved in the binding of alanine, which has no significant effect on that of the two other substrates. On the other hand, in H199A, an increased K_m for UDP-MurNAc was accompanied by an increased K_m for alanine, thus suggesting that the binding of alanine is dependent on that of UDP-MurNAc. Similarly the increase of K_m values for both ATP and UDP-MurNAc in N296A, K130A and N293A suggested that the binding of UDP-MurNAc is dependent on that of ATP. Similar variations were observed in glutathione synthetase (Hara *et al*., 1995). Presumably in both the Mur and glutathione synthetases, substrate binding is an ordered process as established in glutamine synthetase (Meek & Villafranca, 1980), in D-alanine:D-alanine ligase (Mullins *et al*., 1990), and more recently in MurF (Anderson *et al*., 1996). The variations of K_m values observed here for the substrates of MurC suggested that the binding-order would be ATP, UDP-MurNAc, and alanine.

The MurC activity of the wild-type overproducing strain observed after IPTG induction was 1500-fold higher than in the plasmidless strain (*ca*. 1 nmol min^{-1} mg^{-1}; Liger *et al*., 1991). In the absence of IPTG induction, it was still 30-fold higher than in the plasmidless strain, owing presumably to a high plasmid copy number and to a residual transcription under these conditions. By assuming that the 1500/30 ratio was also valid for the overproduced mutated proteins, an upper value of the *in vivo* level of MurC activity in the complementation assays carried without IPTG can be estimated. In N293A and N296A, it would be higher than the basal wild-type level and would thus explain their positive complementation, their normal morphology, and peptidoglycan cell content. It was not possible to conclude whether these residues were essential or not. To test the real effect of their mutagenesis, future work would imply the transfer of the mutated *murC* genes as a unique copy on the chromosome. On the other hand, the very low activity of strains E174A and D351A was clearly reflected by their low peptidoglycan content, their abnormal morphology, and the absence of complementation, and stressed the essential character of residues E174 and D351. Mutants K130A, H199A, and R327A are borderline cases. A slight complementation was observed with K130A, although its MurC activity was less than 5% of that of the wild-type plasmidless strain. This could indicate the minimal level of activity necessary for bacterial cell survival. When estimated on the basis of the 1500/30 ratio, the possible *in vivo* levels of MurC activity in both H199A or R327A were at most 2-fold higher than or similar to the basal wild-type value, respectively. In H199A, the true level is sufficient for complementation and a normal morphology but not quite high enough to insure a normal peptidoglycan content. Presumably, the high K_m value for UDP-MurNAc versus its pool value leads to a reduced activity. Similarly, in R327A, the observed effects (no complementation, ovoid shape, and low peptidoglycan content) could be due to a greatly reduced *in vivo* MurC activity owing to the very high K_m for alanine versus its pool value.

ADDENDUM

While this paper was under review, Eveland *et al*. (1997) published the comparison of the amino acid sequences of MurC, -D, -E, -F, and FolC. The alignment presented allowed them to define four regions of homology, which contained essentially the same invariant amino acids as those described in the present work. The main differences between their results and ours are their overlook of the conserved aspartyl residues in the N- and C-terminal parts, and the claim of a region of homology (sequence 227−240 in MurC) containing a concentrated set of acidic residues (however not conserved in all the sequences). Taking MurF as a model, they prepared the E158A and H188A mutant proteins, equivalent to E174A and H199A, respectively, for MurC. In a standard MurF assay, the mutant proteins displayed 0.02% and 0.5% relative activities, respectively. These data are therefore in agreement with those obtained with MurC by ourselves. The kinetic parameters of the mutant MurF proteins were not determined.

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