The peptidoglycan of *Thermotoga maritima*, an extremely thermophilic eubacterium, was shown to contain no diaminopimelic acid and approximate amounts of both enantiomers of lysine (Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B., and Stetter, K. O. (1986) *Arch. Microbiol.* 144, 324–333). To assess the possible involvement of the MurE activity in the incorporation of d-lysine, the *murE* gene from this organism was cloned in *Escherichia coli*, and the corresponding protein was purified as the C-terminal His$_6$-tagged form. In *vitro* assays showed that d-lysine and meso-diaminopimelic acid were added to UDP-N-acetylmuramoyl-dipeptide with 25 and 10% efficiencies, respectively, relative to l-lysine. The purified enzyme was used to synthesize the l- and d-lysine-containing UDP-N-acetylmuramoyl-tripeptides; chemical analysis revealed an unusual structure for the d-lysine-containing nucleotide, namely acylation of the ε-amino function of d-lysine by the d-glutamyl residue. In *vitro* assays with MurF and MraY enzymes from *T. maritima* showed that this novel nucleotide was not a substrate for MurF but that it could be directly processed into tripeptide lipid I by MraY, thereby substantiating the role of MurE in the incorporation of d-lysine into peptidoglycan.

Peptidoglycan is a giant macromolecule composed of alternating N-acetylgalactosamine and N-acetylmuramyl residues cross-linked by short peptides. Its biosynthesis is a complex two-stage process. The first stage consists of the formation of the disaccharide-pentapeptide monomer unit, whereas the second stage concerns the polymerization reactions (1). The assembly of the peptidoyl part of the monomer unit is ensured by a series of enzymes (MurC, MurD, MurE, and MurF) called the Mur synthetases. It has been shown that the Mur synthetases constitute a family of enzymes with common mechanistic and structural features (2, 3).

Synthetase MurE catalyzes the addition of the third amino acid residue of the peptidyl chain. This residue, generally a diaminoc acid, varies among the bacterial species: meso-diaminopimelic acid (meso-A$_2$pm) for most Gram-negative bacteria and bacilli, l-lysine for most Gram-positive bacteria, l-ornithine, meso-lanthionine, l-A$_2$pm, l-diaminobutyric acid, l-homoserine, etc. in particular species (4). In many organisms, the third residue is involved in the cross-linking of the macro-
molecule; in those cases, the incorporation by MurE of a “wrong” amino acid results in cell lysis (5). Therefore, MurE must be endowed with a high specificity to select the “right” amino acid among closely related amino acids that coexist within the cell. Recently, the crystallization of MurE from *Escherichia coli* allowed Gordon et al. (6) to decipher the structural bases for this high specificity.

*Thermotoga maritima* is a Gram-negative, extremely thermophilic bacterium isolated from geothermally heated sea floors (7). The analysis of its peptidoglycan revealed the absence of A$_2$pm and the presence of both enantiomers of lysine. In this paper, we describe the properties of purified MurE from this bacterial species; in particular, we demonstrate that it is capable of adding l- and d-lysine to UDP-N-acetylmuramyl (MurNAc)-l-Ala-d-Glu with comparable efficiencies but in different ways. The explanation of this finding in terms of known consensus sequences in MurE proteins is discussed. Moreover, we bring evidences of its physiological relevance by showing that: (i) the novel d-lysine-containing nucleotide is a substrate for MraY from *T. maritima in vitro* and (ii) the overexpression of *T. maritima murE* in *E. coli* results in important lysine incorporation into the peptidoglycan of the host.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction enzymes and synthetic oligonucleotides were obtained from New England Biolabs and MWG-Biotech, respectively. Vector pET2160 was constructed from pET21d (Novagen) according to Caravano et al. (8). UDP-MurNAc (9), UDP-MurNAc-l-Ala-d-Glu (10), UDP-MurNAc-l-Ala-d-[14C]Glu (11), UDP-MurNAc-l-[14C]Ala-γ-d-Glu-l-Lys-d-Ala-d-Ala (12), meso-A$_2$pm (13), and *E. coli* MurC (14) and MurD (15) were prepared according to the published procedures. l-Lysine and d-lysine were purchased from Fluka; their enantiomeric purity was confirmed by the measurement of their optical rotation and by amino acid analysis after reaction with L-lysine O-benzyl carbamate (LBN). N$^\text{v}$-Dinitrophenyl (DNP)-l-lysine was purchased from Sigma; N$^\text{v}$-DNP-l-lysine was obtained by trifluoroacetic acid treatment of N$^\text{v}$-DNP, N$^\text{v}$-t-butyloxycarbonyl-l-lysine, itself synthesized by dinitrophenylation of N$^\text{v}$-t-butyloxycarbonyl-l-lysine.

**Bacterial Strains and Growth Conditions**—*E. coli* strains BL21(DE3)/pLysS (Novagen) and DH5α (Invitrogen) were used as hosts for the plasmids as well as for the overproduction of the MurE and MurF enzymes. The *murE* thermosensitive strain PC2336 was obtained from the Phagenex collection (Department of Molecular Cell Biology, State University of Utrecht, Utrecht, The Netherlands), and its complementation by plasmids was tested as described previously (16). 2YT medium (17) was used for growing cells, and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. The antibiotics were used at the following concentrations: ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml).

**General DNA Techniques and E. coli Cell Transformation**—Polymerase chain reaction amplification of DNA was performed in a Thermocycler 60 apparatus (Bio-med) using an Expand High Fidelity PCR sys-

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11 The abbreviations used are: A$_2$pm, 2,6-diaminopimelic acid; DNP, 2,4-dinitrophenyl; MurNAc, N-acetylmuramoyl; HPLC, high pressure liquid chromatography.

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**The MurE Synthetase from *Thermotoga maritima* Is Endowed with an Unusual*** d-Lysine Adding Activity**

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ter (Roche Applied Science); DNA fragments were purified using the Wizard purification system. Plasmid isolations were carried out using the Nucleospin® plasmid extraction system (Macherey-Nagel). Standard procedures were used for endonuclease digestions, ligation, and agarose electrophoresis (18, 19). E. coli cells were made competent and transformed with plasmid DNA using the method of Daget and Ehrlich (20).

Cloning of the T. maritima murE Gene and Plasmid Construction—Standard procedures for molecular cloning were used (19). The T. maritima murE gene was amplified by PCR from the chromosome of strain MSB8; for this purpose, two primers containing a BspHI site (shown in bold type) 5’ to the initiation codon (underlined), 5’-TCTATCATGAAATATATCAACTATCGTGTGC-3’ and a BamHI site (in bold type) 3’ to the gene without its stop codon, 5’-AGATGGATCC-TTGGGCGTATTTCCTCCCCTTCAGC-3’, were employed. The amplified material was digested by BspHI and BamHI and inserted between the compatible Ncol and BgIII sites of vectors pET2160 (T7 promoter) (8) and pTrcHis60 (trc promoter) (21), generating plasmids pABO6 and pABO7, respectively, that code for T. maritima MurE with an ArgSer-(His)6 C-terminal extension. The constructions were verified by DNA sequencing (MWG-Biotech). Plasmids pABO6 and pABO7 were transformed into E. coli strains BL21(DE3)/pLysS and DH5α, respectively, for expression.

Purification of the MurE Enzyme—BL21(DE3)/pLysS/pABO6 cells were grown exponentially at 37 °C in 2YT medium containing ampicillin and chloramphenicol (700 ml of culture). When the optical density reached 0.4, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and growth was continued for 4 h (final optical density, 3.5). The cells were harvested at 4 °C, and the pellet was washed with cold 20 mM phosphate buffer, pH 7.2, 1 mM dithiothreitol (buffer A; 15 ml). They were resuspended in buffer A (6 ml) and disrupted by sonication in the cold using a Bioblock VibraCell 72412 sonicator. The resulting suspension was centrifuged at 4 °C for 30 min at 200,000 × g with a Beckman TL100 apparatus, and the pellet was discarded. The supernatant was kept at −20 °C.

The His6-tagged MurE protein was purified on Ni2+-nitrotetracate-agarose following the manufacturer’s recommendations (Qiagen). All of the operations were performed at 4 °C. The supernatant was mixed with the polymer (1 ml) previously washed with buffer A containing 0.3 M KCl and 10 mM imidazole. The suspension was stirred gently for 2 h in a test tube rotator. Elution was performed with a discontinuous imidazole gradient (20–200 mM) in buffer A containing 0.3 M KCl (the protein was eluted between 60 and 150 mM imidazole). The fractions of interest were pooled and dialyzed against buffer A. The amount of protein recovered, determined by quantitative amino acid analysis, was 3.7 mg (concentration, 0.73 mg/ml). The purity on SDS-PAGE was estimated to be >90%. The enzyme was stored at −20 °C in the presence of 10% glycerol for more than a year without a loss of activity.

Cloning of the murF Gene and Purification of the MurF Enzyme—The same techniques were used for the cloning of the murF gene and the purification of the MurF protein from T. maritima with a His, N-terminal extension. From 1 liter of culture, 18 mg of protein (concentration, 2.05 mg/ml) with a >90% purity were obtained.

Purification of the MraY Enzyme—The MraY enzyme from T. maritima was purified from membranes of E. coli CD43(DE3) cells overexpressing the T. maritima mraY gene by using the same protocol as the one described for the Bacillus subtilis enzyme (12).

Protein Monitoring—Protein concentrations were determined by quantitative amino acid analysis with a Hitachi L8800 analyzer (Scien Tec) after hydrolysis of samples in 6 M HCl at 105 °C for 24 h.

MurE Assay—The MurE activity was assayed by measuring the formation of UDP-MurNAc-l-Ala-γ-D-[14C]Glu-l-Lys in mixtures (final volume, 50 μl) containing 0.1 mM ethanamine-HCl, pH 9.4, 15 mM MgCl2, 5 mM ATP, 100 μM UDP-MurNAc-l-Ala-d-[14C]Glu (420 Bq), 200 μM l-lysine, and enzyme (15 μl of an appropriate dilution in buffer A). After 30 min at 68 °C, the reaction was stopped by the addition of 10 μl of glacial acetic acid. The mixture was lyophilized and taken up in 100 μl of 50 mM sodium phosphate, 7.2 mM sodium hexane sulfonate, pH 2.5, acetonitrile 98.5:1.5 (v/v), and radioactive substrate and product were separated on a Nucleosil 100C18 5 μm column (150 × 4.6 mm; Alltech-France) with the same mixture at 0.6 ml/min as a mobile phase.

Detection was performed with a radioactive flow detector (model LB-506-C1; Berthold) using the Quicksafe Flow 2 scintillator (Zinsser Analytic) at 0.6 ml/min. Quantification was carried out with the Winflow software (Berthold).

For the determination of the kinetic constants, the same assay was used with various concentrations of one substrate and fixed concentrations of the others. In all cases, the substrate consumption was <20%, the linearity being ensured within this interval even at the lowest nucleotide concentration. The data were fitted to the equation $v = V_{max}S/(K_m + S)$ or $v = V_{max}S/(K_m + S + S^2/K_c)$ using the MDFFit software developed by M. Desmadri (IBBMC, Orsay, France). Identical conditions were used when d-Lys or l-Orn were assayed as the amino acid substrate; with meso-Apm, the mobile phase used was 50 mM ammonium formate, pH 3.9 (22).

Preparation of l- and d-Lysine-containing UDP-MurNAc-tripeptides—The reaction mixtures (final volume, 1 ml) contained 0.1 mM ethanamine-HCl, pH 9.4, 15 mM MgCl2, 5 mM ATP, 500 μM UDP-MurNAc-l-Ala-d-Glu, 3 mM l- or d-lysine, and enzyme from T. maritima (7.3 μg). After 1.5 h at 68 °C, they were lyophilized, and each nucleotide product was purified by filtration on a Sephadex G-25 column (115 × 2 cm) in water (23), followed by HPLC on a Vydac 218TP1010 column (250 × 10 mm; eluent A, 0.05% trifluoroacetic acid; eluent B, 0.035% trifluoroacetic acid, methanol 4:1; gradient, 100% A for 5 min, 0–100% B for 20 min, 100% B for 10 min, at 2.5 ml/min). The yields were determined by quantitative amino acid analysis. The nature of each product as lysine-containing UDP-MurNAc-tripeptide was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using the conditions of Girardin et al. (24).

The d-lysine-containing UDP-MurNAc-tripeptide was also produced by MurE from E. coli (6). In this case, the buffer was 0.1 M Tris-HCl, pH 8.6, and the MgCl2 concentration was 100 mM.

Preparation of Radiolabeled UDP-MurNAc-tripeptides—The reaction mixtures (final volume, 70 μl) contained 0.1 mM Tris-HCl, pH 8.6, 10 mM MgCl2, 5 mM ATP, 0.90 mM UDP-MurNAc-l-Ala-d-[14C]Glu (0.4 MBq), 5 mM d-Glu, and MurC and MurD from E. coli (12 and 20 μg, respectively). After 5 h at 37 °C, 10 mM (final concentration) l- or d-Lys and 3.5 μg of MurE from T. maritima were added, the temperature was raised to 68 °C, and the incubation was continued for 3 h. The labeled nucleotides were purified as described above.

Enzymatic Determination of Lysine Stereochemistry—Each nucleotide (20 nmol) was hydrolyzed by 6 M HCl at 95 °C for 16 h. After evaporation of the acid, the hydrolyzates were taken up in 50 μl of 0.5 M sodium acetate, pH 6.0, and incubated with 300 μg of l-lysine decarboxylase (Worthington) at 37 °C for 3 h. The reaction mixtures were lyophilized, taken up in 67 mM trisodium citrate-HCl buffer, pH 2.2, and injected into the amino acid analyzer.
**MurE from T. maritima**

**Dinitrophenylation**—Each nucleotide (40 nmol in 50 µl of water) was mixed with 25 µl of 0.1 M dinitrofluorobenzene in ethanol and 10 µl of triethylamine. After 30 min at 60 °C in the dark, the mixtures were evaporated, and the residues were taken up in 200 µl of 6 M HCl and extracted with ether to remove dinitrophenol. They were then hydrolyzed at 95 °C for 16 h. After evaporation of the acid, the hydrolyzates were extracted with ether to remove dinitrophenol. They were then hydrolyzed at 95 °C for 16 h. After evaporation of the acid, the hydrolyzates were extracted with ether to remove dinitrophenol and 10 µl of triethylamine. After 30 min at 60 °C in the dark, the mixtures were analyzed by HPLC with on-line scintillation counting as described for MurE, except that the mobile phase was 0.1M ammonium acetate, pH 6.0.

**MraY Assay**—The MraY activity was assayed as previously described (25). Aliquots were hydrolyzed (6 M HCl, 95 °C, 16 h), and the hydrolyzates were injected into the amino acid analyzer before and after incubation with L-lysine decarboxylase, thereby allowing the quantitation of the amino acid pools.

**Isolation of Peptidoglycan from E. coli Cells**—DH5α/pABO7 cells were grown as described above, without and with isopropyl-β-D-thiogalactopyranoside induction. Induction was initiated at an optical density of 0.34 and continued for 2 h. Peptidoglycan was purified by hot SDS treatment, followed by successive incubations with pancreatin, pronase, and trypsin, as already described (25). Aliquots were hydrolyzed (6 M HCl, 95 °C, 16 h), and the hydrolyzates were injected into the amino acid analyzer before and after incubation with L-lysine decarboxylase, thereby allowing the quantitation of the amino acid pools.

**Extraction of the Free Amino Acids from T. maritima**—T. maritima cells were grown as described in the literature (7). After harvesting, 0.6 g of wet cell pellet was put into 25 ml of boiling water and heated at 100 °C for 30 min. After chilling in ice water, cold trichloroacetic acid was added to a final concentration of 5%. The resulting suspension was kept at 4 °C for 20 min before centrifugation (15,000 × g for 20 min). The resulting supernatant was extracted three times with ether to remove trichloroacetic acid, adjusted to pH 7 by sodium hydroxide, and lyophilized. The residue was taken up in 2 ml of water, and aliquots were injected into the amino acid analyzer before and after incubation with L-lysine decarboxylase, thereby allowing the quantitation of the amino acid pools.

**RESULTS**

**Overproduction and Purification of MurE from T. maritima**—The murE gene from T. maritima chromosome was cloned into vector pET2160, which allows the expression of proteins with a C-terminal His tag (8). The MurE protein was expressed in E. coli and purified by affinity chromatography. The purification was monitored: (i) by SDS-PAGE and (ii) by an assay of addition of L-lysine to UDP-MurNAc-dipeptide; this amino acid was chosen because T. maritima peptidoglycan was shown to contain lysine and not A2pm (7). The recombinant protein exhibited a molecular mass of ~56 kDa as judged by SDS-PAGE. The yield was 5.3 mg per liter of culture.

**Enzymatic Properties**—The behavior of the L-lysine adding activity as a function of temperature, pH, and magnesium concentration was studied. Curves (Fig. 1) displayed the usual bell shape, with optimal values of 68 °C, 9.4, and 15 mM, respectively. Examination of the first curve (Fig. 1A) indicated that at 37 °C, the temperature at which the enzyme was expressed, the activity was ~25% of the optimal value.

**Alternative Amino Acid Substrates and Kinetic Parameters**—With L-lysine as the amino acid substrate, the V<sub>max</sub> of the enzyme was 56 µmol/min/mg (Table 1). However, because a strong inhibition by excess UDP-MurNAc-dipeptide was observed (Table 1 and Fig. 2A), this value is only theoretical. To have a compromise between enzyme saturation and substrate inhibition, the fixed value of this substrate was set at 0.2 mM. The kinetic parameters found for the amino acid substrates were therefore apparent, but they could be compared with one another. Besides L-lysine, T. maritima MurE was shown to be capable of adding D-lysine and meso-A<sub>2</sub>pm to UDP-MurNAc-dipeptide. With respect to V<sub>max</sub>/K<sub>m</sub> ratio for L-lysine, those for the latter amino acids were 4- and 10-fold lower, respectively. A weak but significant addition of L-ornithine was also detected (V<sub>max</sub>/K<sub>m</sub> ratio: 460-fold lower than that for L-lysine). The kinetic parameters for all substrates are shown in Table 1, and representative curves are shown in Fig. 2.

**FIGURE 1.** Curves of the L-lysine adding activity as a function of temperature (A), pH (B), and Mg<sup>2+</sup> concentration (C). The concentrations of ATP, UDP-MurNAc-L-Ala-D-Glu, and L-lysine are 5 mM, 100 µM, and 200 µM, respectively. 100% activity corresponds to 1 µmol/min/mg.

**TABLE 1**

| Substrate | Apparent K<sub>m</sub> | Apparent V<sub>max</sub> | Apparent V<sub>max</sub>/K<sub>m</sub> |
|-----------|----------------------|------------------------|-------------------------------|
| ATP<sup>a</sup> | 3.6 ± 0.3 | 25 ± 1 | 6.9 |
| UDP-MurNAc-L-Ala-D-Glu<sup>a</sup> | 0.45 ± 0.10 | 56 ± 5 | 120 |
| L-lysine | 2.8 ± 0.2 | 27 ± 2 | 9.6 |
| D-lysine | 1.7 ± 0.3 | 41 ± 0.3 | 2.4 |
| meso-A<sub>2</sub>pm | 4.8 ± 0.7 | 48 ± 0.3 | 1.0 |
| L-ornithine | 27 ± 1 | 0.57 ± 0.01 | 0.021 |

<sup>a</sup> Determined with L-lysine as the amino acid substrate.

<sup>a</sup> The kinetic parameters for this substrate were determined by fitting the data to the equation v = v<sub>max</sub>S/(K<sub>m</sub> + S + S<sup>2</sup>/K<sub>c</sub>); the K<sub>c</sub> value found was 0.066 ± 0.012 mM.
Structure of the Lysine-containing UDP-MurNAc-tripeptides Produced by MurE from T. maritima—The UDP-MurNAc-tripeptides formed upon the addition of either enantiomer of lysine were purified, and their chemical identity was confirmed by amino acid analysis and mass spectrometry (Table 2). In analytical HPLC (22), the nucleotide formed from L-lysine had the same retention time as authentic UDP-MurNAc-L-Ala-D-Glu-L-Lys, whereas the one formed from D-lysine had a higher value (Table 2). L-Lysine decarboxylase digestion of acid hydrolyzates showed that the stereochemistry of lysine in the nucleotides was the same as that of the starting amino acids (Table 2). To identify the amino function of lysine (α or ε) involved in the isopeptide bond with the γ-carboxyl function of D-Glu, both nucleotides were dinitrophenylated and hydrolyzed. TLC in two different solvent systems showed that in the L-lysine-containing nucleotide, lysine was acylated on its α-amino function, whereas in the D-lysine-containing nucleotide, it was acylated on its ε-amino function (Table 2 and Fig. 3).

The UDP-MurNAc-tripeptides as Possible Substrates for MurF and MraY—We wanted to check whether the UDP-MurNAc-tripeptides were substrates for the downstream enzymes MurF and MraY from T. maritima. For this purpose, both enzymes were purified, and the nucleotides were synthesized in a radioactive form. As expected, the L-lysine-containing nucleotide was a substrate for MurF, whereas the D-lysine-containing nucleotide was not. With MraY, the D-lysine-containing UDP-MurNAc-tripeptide was processed as efficiently as the conventional UDP-MurNAc-pentapeptide. The L-lysine-containing UDP-MurNAc-tripeptide was a substrate, too, but to a lesser extent (Table 3).

MurE from T. maritima Is Functional in Vivo—The expression of the T. maritima murE gene from either the pABO6 or the pABO7 plasmid (T7 and trc promoters, respectively) in E. coli cells slowed down the growth rate but did not result in cell lysis. To determine whether the expression had provoked an incorporation of lysine into peptidoglycan,
**MurE from T. maritima**

**TABLE 3**

**Effect of MurF and MraY from T. maritima on the UDP-MurNAc-peptides**

The activity of purified MurF and MraY on L- or D-lysine-containing UDP-MurNAc-peptides was tested as described under “Experimental Procedures.”

| Compound                                | Activity*  |
|-----------------------------------------|------------|
| UDP-MurNAc-tripeptide (D-Lys)           | 230 ± 10   |
| UDP-MurNAc-tripeptide (L-Lys)           | ND*        |
| UDP-MurNAc-tripeptide (D-Lys)           | 34 ± 0.2   |
| UDP-MurNAc-tripeptide (L-Lys)           | 21 ± 2     |
| UDP-MurNAc-pentapeptide (L-Lys)         | 19 ± 1     |

*Mean ± S.D. of a triplicate determination.

*ND, not detected (no product formation after 4 h with 3 μg of MurF).

DH5α/pABO7 and DH5α/pTrcHis60 cells were grown in the absence or presence of isopropyl β-D-thiogalactopyranoside, and their peptidoglycan was purified and analyzed. In wild type DH5α/pTrcHis60 cells, peptidoglycan constituents muramic acid, glucosamine, alanine, glutamic acid, and lysine were present in a ratio of 0.9:1:1:2:1:1:0:1, respectively. The 10% lysine found originate from Braun’s lipoprotein, whose C-terminal lysine is linked to A2pm residues (5, 26). In DH5α/pABO7 cells, the same constituents were as follows: 0.9:1:2:1.4:4:6:0:6 without induction and 0.9:1:1.8:1.3:0:5:0:7 with induction. Therefore, in transformed cells a large incorporation of lysine has occurred in the macromolecule, with half of the A2pm residues being replaced by lysine. This showed that the murE gene was functional in this heterologous background.

We also tested whether the T. maritima murE gene could complement the defect of the E. coli thermosensitive murE mutant PC2336. The result was negative (no complementation) with both the pABO6 and pABO7 plasmids; furthermore, transformants at the permissive temperature were obtained only after 48 h. This indicated that the expression of the T. maritima murE gene was toxic in such a genetic background where the level of the A2pm adding MurE enzyme originating from the chromosome of the host is greatly altered. This finding suggested that in DH5α/pABO7 cells, both the E. coli and T. maritima MurE enzymes contribute to the incorporation of A2pm into the peptidoglycan that is required to maintain cell viability.

**Intracellular Amino Acid Pools in T. maritima**—To explain the absence of A2pm in T. maritima peptidoglycan, although this amino acid is a fairly good substrate for MurE in vitro, we measured the amino acid pools in this organism. Lysine and A2pm were present at 2000 and 2.3 nmol/g wet weight, respectively. The proportion of D isomer in the lysine pool was 17%.

**DISCUSSION**

Because of its thermophilic character, T. maritima has been the subject of a great deal of work. However, studies on the peptidoglycan of this species and its metabolism are scarce. In their initial work on the organism, Huber et al. (7) determined the chemical composition of the macromolecule; more recently, the crystal structure of MurC was solved as part of a structural genomics program (27).

At first sight, the composition of the peptid part of T. maritima peptidoglycan (Glu, 1.00; Ala, 1.43; Lys, 0.89) (7) seems common. However, the presence of both enantiomers of lysine in a ~1:1 molar ratio (7) is intriguing. D-Lysine is rarely found in peptidoglycan. It is present in that of Butyribacterium rettgeri (28); the residue in position 3 is L-ornithine, and D-lysine serves as a cross-linking amino acid between the α-carboxyl of D-Glu of a peptide subunit and the carboxyl of D-Ala of another subunit. In this species, D-ornithine also plays this role, the ratio of D-lysine to D-ornithine bridges being 2:1.

In the case of T. maritima, the fact that (L + D)-lysine was not in excess with respect to D-Glu led us to suspect that the d enantiomer was not present as a cross-linking amino acid but in the main peptide chain along with the more usual L enantiomer. In other words, MurE would be capable of using both enantiomers of lysine as substrates. To verify this assumption, we cloned the murE gene and purified the corresponding protein on the basis of the L-lysine adding activity measurement. In vitro essays with the purified enzyme showed that it was able to add not only L-lysine but also D-lysine and meso-A2pm to UDP-MurNAc-L-Ala-D-Glu. The chemical analysis of the lysine-containing products showed that the starting stereoisomer (L or d) had really been added; this rules out the possibility of artifacts due either to traces of the other stereoisomer or to a racemase copurified with MurE. The comparison of the specificity constants for the three amino acids, which vary over only 1 order of magnitude, indicates that the addition of L-lysine and meso-A2pm are not side reactions of the enzyme, as is the case for L-ornithine, and explains why D-lysine is present in the peptidoglycan of the bacterium. The absence of meso-A2pm was more intriguing; indeed, this amino acid was likely to be present in T. maritima because all the genes encoding for the enzymes of its metabolic pathway exist (www.tigr.org). It can be explained by a low intracellular concentration; according to our results, the intracellular pool of A2pm is 720- and 150-fold lower than those of L- and D-lysine, respectively.

The almost total lack of stereospecificity of T. maritima MurE toward lysine is unexpected. Diamino acid substrates such as meso-A2pm or L-lysine have two recognition sites: the proximal site, which participates in the peptide bond, and the distal site, which carries the nonreacting amino function (29). In the peptide subunit of peptidoglycan, the proximal recognition site of meso-A2pm and L-lysine consists in the L-asymmetric center, and the distal recognition site in the D (meso-A2pm) or achiral (L-lysine) center (Fig. 3). We assumed that in D-lysine, the proximal and distal sites would be the achiral and d centers, respectively. In other words, D-lysine would not be acylated on its α-amino function but on its ε one. This turned out to be the case. Therefore, in T. maritima MurE, the substrate for the reacting moiety of the diaminoc acid accepts L- or achiral carbons, whereas the subsite for the nonreacting moiety accepts achiral or d carbons (Fig. 3).

It should be mentioned that the Km values of Table 1 were determined at pH 9.4, where the α-amino group of lysine (pK = 8.90) is largely deprotonated and the ε-amino group (pK = 10.28) is largely protonated (30). This raises the question of whether the concentration of the nucleophile in the reaction is that of the added lysine. To address this question, the Km values were also determined at pH 8.0, where the α-amino group is largely protonated and the ε-amino group almost totally protonated. The values found were 0.65 ± 0.30 and 1.0 ± 0.3 mM for L- and D-lysine, respectively, indicating that the affinity for the amino acid substrate does not depend on the concentration of the deprotonated form. (The corresponding Vmax values were 0.56 ± 0.13 and 0.36 ± 0.04 μmol/min/mg, respectively.)

The examination of the crystal structure of MurE from E. coli had revealed the existence of a binding pocket for the distal recognition site of meso-A2pm (Fig. 4A) (6). Upon alignment of a few MurE sequences, two consensus sequences had been proposed for the recognition of meso-A2pm and L-lysine (6). Later, the alignment of a larger number of MurE sequences allowed a better definition of the two conserved motifs (31), characterized by the tetrapeptide sequences DNPR (meso-A2pm recognition) and D(D,N)P(N,A) (L-lysine recognition) (Fig. 4B). The main difference between the two sequences is the arginyl residue, which...
is hydrogen-bonded to the carboxyl group linked to the D carbon of meso-A_{2pm} (Fig. 4A) (6). For T. maritima, the corresponding sequence, DDPR, is obviously a meso-A_{2pm} recognition one (Fig. 4B). Therefore, it is not surprising that it accepts the D carbon of meso-A_{2pm} and D-lysine. Interestingly, D-lysine was shown to be a better substrate for E. coli MurE in vitro than the L isomer; the relative specific activity of incorporation for meso-A_{2pm}, D-lysine, and L-lysine was 100, 0.6, and < 0.1, respectively (32). However, the arrangement of the D-Glu-D-Lys bond had not been determined. In the course of the present work, we have isolated and analyzed the D-lysine-containing UDP-MurNAc-tripeptide produced by MurE from E. coli; we have found that this enzyme catalyzes the acylation of the ε-amino function of D-lysine as T. maritima MurE does. In this regard, E. coli and T. maritima MurE enzymes share the same specificity for the distal recognition site of meso-A_{2pm}. As far as the latter enzyme is concerned, the main question mark is its high efficiency toward L-lysine, whereas the former hardly accepts it as a substrate. The resolution of the three-dimensional structure of T. maritima MurE would help answer this question.

An important issue is the physiological relevance of the results obtained with D-lysine: is MurE actually responsible for the incorporation of D-lysine into peptidoglycan in vivo? If yes, is the D-lysine-containing tripeptide elongated by MurF, or is it transferred as such by downstream enzymes MraY, MurG, and glycosyltransferases. In the present work, we demonstrated that the D-lysine-containing UDP-MurNAc-tripeptide was a good substrate for T. maritima MraY, as good as the conventional UDP-MurNAc-pentapeptide, yielding tripeptide lipid I (Table 3). Such a behavior is not unprecedented; in E. coli, the formation of tripeptide lipid II from UDP-MurNAc-tripeptide was reported in a cell-free system (33), implying that the meso-A_{2pm}-containing UDP-MurNAc-tripeptide is a substrate for MraY (and MurG) from this species. Taking into account the usual lack of specificity of MurG and of the glycosyltransferases for the peptide part of the lipid intermediates, it is reasonable to consider that the D-lysine-containing tripeptide is incorporated into the peptidoglycan of T. maritima, as it is the case for the meso-A_{2pm}-containing tripeptide into the peptidoglycan of E. coli (33).

At this stage, another question arises: does the D-lysine-containing peptide stem remain as a monomer, or is it a substrate for transpeptidas, thereby being incorporated into dimers? In the latter case, it could be used only as an acceptor because of the absence of the D-Ala-D-Ala moiety. Only the elucidation of the structure of T. maritima peptidoglycan will solve this problem.

It should be mentioned that in the case of the L-lysine adding enzyme from Staphylococcus aureus, its overproduction in E. coli had resulted in cell lysis. This was explained by a strong incorporation of lysine into the peptidoglycan of the host (55% with respect to A_{2pm} in cells collected prior to lysis) (5). However, in the present work, we were surprised that no lysis of host cells occurred during the overproduction of MurE from T. maritima; only a decrease of the growth rate was observed. This might raise questions about the functionality of the enzyme. To address this problem, we analyzed the peptidoglycan from the overproducing T. maritima and examined whether the L-lysine-containing peptide stem remained as a monomer or was incorporated into dimers. The fact that no lysis occurred is presumably due to the ability of the enzyme...
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to also add meso-A2pm (Table 1), contrary to that from S. aureus, which does not accept this amino acid as a substrate. With *S. aureus* MurE, the increasing incorporation of lysine ends in cell lysis, whereas with the *T. maritima* enzyme, a low but constant A2pm/Lys ratio ensures viability. Clearly, a balance between the A2pm adding (T. maritima + chromosomal *E. coli*) and lysine adding (T. maritima) activities is necessary to achieve this ratio, as shown by the absence of complementation at 42 °C and the very slow growth of the transformants at 30 °C observed with the murE thermosensitive strain.

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REFERENCES

1. van Heijenoort, J. (2001) *Nat. Prod. Rep.* **18**, 503–519
2. Eveland, S. S., Pompliano, D. L., and Anderson, M. S. (1997) *Biochemistry* **36**, 6223–6229
3. Bouhss, A., Mengin-Lecreulx, D., Blanot, D., van Heijenoort, J., and Parquet, C. (1997) *Biochemistry* **36**, 11556–11563
4. Schleifer, K. H., and Kandler, O. (1972) *Bacteriol. Rev.* **36**, 407–477
5. Mengin-Lecreulx, D., Falla, T., Blanot, D., van Heijenoort, J., Adams, D. J., and Chopra, I. (1999) *J. Bacteriol.* **171**, 4909–5914
6. Gordon, E., Flouret, B., Chantalat, L., van Heijenoort, J., Mengin-Lecreulx, D., and Dideberg, O. (2001) *J. Biol. Chem.* **276**, 10999–11006
7. Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B., and Stetter, K. O. (1986) *Arch. Microbiol.* **144**, 324–333
8. Caravano, A., Mengin-Lecreulx, D., Brondello, J.-M., Vincent, S. P., and Sinaý, P. (2003) *Chem. Eur. J.* **9**, 5888–5898
9. Blanot, D., Auger, G., Liger, D., and van Heijenoort, J. (1994) *Carbohydr. Res.* **252**, 107–115
10. Bertrand, J. A., Auger, G., Martin, L., Fanchon, E., Blanot, D., Le Keller, D., van Heijenoort, J., and Dideberg, O. (1999) *J. Mol. Biol.* **289**, 579–590
11. Michaud, C., Mengin-Lecreulx, D., van Heijenoort, J., and Blanot, D. (1990) *Eur. J. Biochem.* **194**, 853–861
12. Bouhss, A., Crouvoisier, M., Blanot, D., and Mengin-Lecreulx, D. (2004) *J. Biol. Chem.* **279**, 29974–29980
13. van Heijenoort, J., and Bricas, E. (1968) *Bull. Soc. Chim. Fr.* 7, 2828–2831
14. Liger, D., Masson, A., Blanot, D., van Heijenoort, J., and Parquet, C. (1995) *Eur.

A. Boniface, S. Dementin, and D. Blanot, unpublished results.