The Elucidation of the Structure of Thermotoga maritima Peptidoglycan Reveals Two Novel Types of Cross-link*

Audrey Boniface†, Claudine Parquet†, Michel Arthur*‡‡, Dominique Mengin-Lecreulx*‡‡, and Didier Blanot*†‡

From the †Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, Université Paris-Sud, 91405 Orsay, ‡CNRS, UMR 8619, 91405 Orsay, the §Centre de Recherche des Cordeliers, Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Pierre et Marie Curie, UMR S 872, 75006 Paris, the ‡Université Paris-Descartes, UMR S 872, 75006 Paris, and **INSERM, U872, 75006 Paris, France

Thermotoga maritima is a Gram-negative, hyperthermophilic bacterium whose peptidoglycan contains comparable amounts of L- and D-lysine. We have determined the fine structure of this cell-wall polymer. The muropeptides resulting from the digestion of peptidoglycan by mutanolysins were separated by high-performance liquid chromatography and identified by amino acid analysis after acid hydrolysis, dinitrophenylation, enzymatic determination of the configuration of the chiral amino acids, and mass spectrometry. The high-performance liquid chromatography profile contained four main peaks, two monomers, and two dimers, plus a few minor peaks corresponding to anhydro forms. The first monomer was the D-lysine-containing disaccharide-tripeptide in which the D-Glu-D-Lys bond had the usual γ→e arrangement (GlcNAc-MurNAc-L-Ala-γ-D-Glu-e-D-Lys). The second monomer was the conventional disaccharide-tetrapeptide (GlcNAc-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala). The first dimer contained a disaccharide-L-Ala as the acyl donor cross-linked to the α-amine of D-Lys in a tripeptide acceptor stem with the sequence of the first monomer. In the second dimer, donor and acceptor stems with the sequences of the second and first monomers, respectively, were connected by a D-Ala4-α-D-Lys3 cross-link. The cross-linking index was 10 with an average chain length of 30 disaccharide units. The structure of the peptidoglycan of T. maritima revealed for the first time the key role of D-Lys in peptidoglycan synthesis, both as a surrogate of L-Lys or meso-diaminopimelamic acid at the third position of peptide stems and in the formation of novel cross-links of the L-Ala3(α→α)D-Lys3 and D-Ala4(α→α)D-Lys3 types.

Peptidoglycan (or murein) is a giant macromolecule whose main function is the protection of the cytoplasmic membrane against the internal osmotic pressure. It is composed of alternating residues of N-acetylmuramic acid (GlcNAc) and N-acetyl muramiduramic acid (MurNAc) cross-linked by short peptides (1). The composition of the peptide stem in nascent peptidoglycan is L-Ala3-γ-D-Glu2-α-X3-D-Ala3-α-D-Ala3, where X is most often meso-diaminopimelic acid (meso-A2pm) or L-lysin in Gram-negative and Gram-positive species, respectively (2, 3). In the mature macromolecule, the last D-Ala residue is removed. Cross-linking of the glycan chains generally occurs between the carboxyl group of D-Ala at position 4 of a donor peptide stem and the side-chain amino group of the diamino acid at position 3 of an acceptor peptide stem (4–3 cross-links). Cross-linking is either direct or through a short peptide bridge such as pentaglycine in Staphylococcus aureus (2, 3). The enzymes for the formation of the 4–3 cross-links are active-site serine DD-transpeptidases that belong to the penicillin-binding protein (PBP) family and are the essential targets of β-lactam antibiotics in pathogenic bacteria (4). Catalysis involves the cleavage of the D-Ala4-D-Ala5 bond of a donor peptide stem and the formation of an amide bond between the carboxyl of D-Ala4 and the side chain amine at the third position of an acceptor stem. Transpeptidases of the X specificity are active-site cysteine enzymes that were shown to act as surrogates of the PBPs in mutants of Enterococcus faecium resistant to β-lactam antibiotics (5). They cleave the X3-D-Ala4 bond of a donor stem peptide to form 3→3 cross-links. This alternate mode of cross-linking is usually marginal, although it has recently been shown to predominate in non-replicative “dormant” forms of Mycobacterium tuberculosis (6).

Thermotoga maritima is a Gram-negative, extremely thermophilic bacterium isolated from geothermally heated sea floors by Huber et al. (7). A morphological characteristic is the presence of an outer sheath-like envelope called “toga.” Although the organism has received considerable attention for its biotechnological potential, studies about its peptidoglycan are scarce (8–11), and in particular the fine structure of the macromolecule is still unknown. In their initial work, Huber et al. (7) showed that the composition of its peptidoglycan was unusual for a Gram-negative species, because it contained both isomers of lysine and no A2pm. Recently, we purified and studied the properties of T. maritima MurE (12); this enzyme is responsible for the addition of the amino acid residue at posi-
tion 3 of the peptide stem (13, 14). We demonstrated that *T. maritima* MurE added in vivo L- and D-Lys to UDP-MurNAc-L-Ala-D-Glu. Although L-Lys was added in the usual way, yielding the conventional nucleotide UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys containing a D-Glu (γ→α)-L-Lys amide bond, the D-isomer was added in an "up-side-down" manner, yielding the novel nucleotide UDP-MurNAc-L-Ala-γ-D-Glu (γ→ε)-L-Lys. We also showed that the D-Lys-containing nucleotide was not a substrate for *T. maritima* MurF, the subsequent enzyme in the biosynthetic pathway, whereas this ligase catalyzed the addition of dipeptide D-Ala-D-Ala to the L-Lys-containing tripeptide, yielding the conventional UDP-MurNAc-pentapeptide (12).

However, both the L-Lys-containing UDP-MurNAc-pentapeptide and D-Lys-containing UDP-MurNAc-tripeptide were used as substrates by *T. maritima* MraY with comparable efficiencies in vitro (12). This observation implies that the unusual D-Lys-containing peptide stems are likely to be translocated to the periplasmic face of the cytoplasmic membrane and to participate in peptidoglycan polymerization. Therefore, we have determined here the fine structure of *T. maritima* peptidoglycan and we have shown that L-Lys- and D-Lys-containing peptide stems are both present in the polymer, the latter being involved in the formation of two novel types of peptidoglycan cross-link.

**EXPERIMENTAL PROCEDURES**

**Materials and General Procedures**—Mutanolysin, L-lysine decarboxylase, and d-amino acid oxidase were purchased from Sigma. The dinitrophenyl amino acids were from Sigma, except N⁵-dinitrophenyl-L-lysine, which was synthesized according to our published procedure (12). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide was synthesized according to Marfey (15).Lyophilizations were performed with a Labconco Freeze Dry-3 system.

**Preparation of *T. maritima* Peptidoglycan—*T. maritima* cells** were grown as described in the literature (7). Bacterial cells were harvested by centrifugation, and the wet cell pellet (0.4 g) was resuspended in 1 ml of 20 mM potassium phosphate (pH 7.2) containing 1 mM dithiothreitol. Bacteria were disrupted by sonication in ice with a Bioblock Vibrascell 72412 sonicator. The resulting suspension was incubated for 30 min at 60 °C in the presence of 0.5% Triton X-100, and centrifuged at 200,000 × g at 20 °C for 20 min with a Beckman TL100 apparatus. The pellet was washed four times with water, resuspended in 1 ml of 0.1 M ammonium acetate (pH 6.0), and incubated with 1 mg ml⁻¹ egg white lysozyme at 37 °C for 2.5 h. This enzyme was previously shown to release the toga from peptidoglycan (7). After removal of insoluble particles by centrifugation, cold acetone (-20 °C, 2 volumes) was added to the supernatant. Following centrifugation, the amino acid and hexosamine contents of the supernatant and pellet were determined, revealing the presence of the digested peptidoglycan material in the supernatant (280 nmol of GlcNAc), in contrast to the previous report where it was found in the pellet (7).

**Muramidase Digestion of Peptidoglycan and HPLC Purification of the Muropeptides—Acetone supernatant (1 ml) was evaporated under vacuum and resuspended in 100 μl of water. Sixty microliters of 0.2 M potassium phosphate (pH 6.5) and 40 μg of mutanolysin were added to the preparation, which was incubated overnight at 37 °C. The muropeptides were reduced with sodium borohydride for 30 min. The solution was acidified with 10 μl of 20% phosphoric acid and loaded onto a column of ODS-Hypersil (3 μm, 250 × 4.6 mm). Elution was performed at a flow rate of 0.6 mL min⁻¹ with a gradient of acetonitrile applied between 10 and 100 min (eluant A: 0.05% trifluoroacetic acid; eluant B: 0.035% trifluoroacetic acid/acetonitrile 4:1 (v/v)). Elution was continued for an additional 15 min with 100% of eluant B. The compounds were detected at 210 nm. Peaks were collected manually and lyophilized. Integration of the absorption areas of the muropeptide peaks was used to determine the cross-linking index and average chain length, according to Glauner et al. (16).

**Amino Acid and Hexosamine Analysis—**Samples were hydrolyzed in 6 M HCl at 95 °C for 16 h. After evaporation of the acid, the hydrolysates were dissolved in 67 mM trisodium citrate-HCl (pH 2.2) and injected into a Hitachi L8800 analyzer equipped with a 2620MSC-P5 column (ScienceTec).

**Chiral Analysis of Peptidoglycan Amino Acids—**An aliquot of the acetic supernatant was evaporated under vacuum and hydrolyzed as described above. After evaporation of the acid, the hydrolysate was dissolved in 25 μl of water and mixed with 50 μl of 2.5 mM 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (15) in acetonitrile and 10 μl of 1 M sodium bicarbonate. After stirring for 1 h in the dark, the reaction was stopped with 20 μl of 2 M HCl. 40 μl of the resulting solution was diluted with 260 μl of 20 mM ammonium phosphate (pH 5.7) and loaded onto a column of LiChrospher RP-18 (5 μm, 250 × 4.6 mm). Elution was performed at 1 mL min⁻¹ with a gradient of acetonitrile (eluant A: 20 mM ammonium phosphate (pH 5.7); eluant B: same buffer/acetonitrile 2:3 (v/v); gradient: 0 to 100% B for 65 min). The compounds were detected at 340 nm. For the sake of identification and quantification, L- and D-isomers of Ala, Glu, and Lys were derivatized and chromatographed using the same procedure. The retention times were 37.2 min (L-Glu), 39.6 min (D-Glu), 46.9 min (L-Ala), 53.6 min (D-Ala), 63.2 min (L-Lys), and 66.6 min (D-Lys).

**Enzymatic Determination of Lysine Stereochemistry—**Peptidoglycan (20 nmol of Lys) or muropeptide (20 nmol) was hydrolyzed as described above. After evaporation of the acid, the hydrolysate was dissolved in 50 μl of 0.5 M sodium acetate (pH 6.0) and incubated with 300 μg of L-Lys decarboxylase for 3 h at 37 °C. The reaction mixture was lyophilized, dissolved in 67 mM trisodium citrate-HCl buffer (pH 2.2), and injected into the amino acid analyzer.

**Enzymatic Determination of Alanine Stereochemistry—**Muropeptide (20 nmol) was hydrolyzed as described above. After evaporation of the acid, the hydrolysate was dissolved in 50 μl of 20 mM potassium phosphate (pH 7.2) and incubated with 400 μg of d-amino acid oxidase for 30 min at 37 °C. The reaction mixture was diluted 10 times with 67 mM trisodium citrate-HCl buffer (pH 2.2), and aliquots were injected into the amino acid analyzer.

**Dinitrophenylation—**Muropeptide (40 nmol) in 50 μl of water was mixed with 25 μl of 0.1 M dinitrofluorobenzene and 10 μl of triethylamine. After 30 min of incubation at 60 °C in the dark, the mixture was evaporated, and the residue was dissolved in 200 μl of 6 M HCl, extracted with ether to remove dinitro-
Structure of T. maritima Peptidoglycan

phenol, and hydrolyzed at 95 °C for 16 h. After evaporation of the acid, the hydrolysate was dissolved in 100 μl of water/methanol 3:2 (v/v) containing 0.1% phosphoric acid and loaded onto a column of Nucleosil 100C18 (5 μm, 250 × 4.6 mm). Elution was performed with the same mixture at 0.6 ml·min⁻¹. The compounds were detected at 350 nm. For sake of identification and quantification, standards of N°-dinitrophenyl-Lys, N°-dinitrophenyl-Lys, N°-dinitrophenyl-Glu, and N°-dinitrophenyl-Ala were analyzed in the same conditions; the retention times were 19, 26, 53, and 79 min, respectively.

Preparation of Lactoyl Peptides—The non-reduced muropeptides from a muramidase digest were purified by HPLC with the same gradient as for the reduced muropeptides (see above). They were identified by amino acid analysis and MALDI-TOF mass spectrometry. The non-reduced forms of the four main peaks (10 nmol) were lyophilized and dissolved in a mixture of 100 μl of water and 37 μl of 4 M ammonium hydroxide. After 5 h of incubation at 37 °C, the reaction was quenched with 31 μl of acetic acid. The lactoyl peptides obtained were purified by HPLC with the same gradient as above and lyophilized.

MALDI-TOF Mass Spectrometry—Positive and negative spectra were recorded in the reflectron mode with delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm laser. Muropeptide (1 μl at 50–100 pmol·μl⁻¹ in water) was deposited on the plate, followed by 2,5-dihydroxybenzoic acid (1 μl at 10 mg·ml⁻¹ in 0.1% citric acid). After evaporation of water, spectra were recorded at an acceleration voltage of ± 20 kV and an extraction delay time of 200 ns. External calibration was performed using the calibration mixture 1 of the Sequazyme™ peptide mass standards kit (Applied Biosystems) in the positive mode, or a mixture of UDP-MurNAc, UDP-MurNAc-dipeptide, and UDP-MurNAc-pentapeptide in the negative mode.

Tandem Mass Spectrometry—The structure of the purified lactoyl peptides was determined with an electrospray time-of-flight mass spectrometer operating in the positive mode (Qstar Pulsar I, Applied Biosystems) as previously described (17). Briefly, [M+H]⁺ ions were selected on the basis of the m/z value in the high resolution mode, and fragmentation was performed with nitrogen as the collision gas. The collision energy was typically of 25–28 and 50–70 eV for the monomers and dimers, respectively.

RESULTS

Purification and Chemical Composition of the Peptidoglycan of T. maritima—Peptidoglycan was extracted according to the procedure published by Huber et al. (7). Bacterial envelopes were treated with lysozyme to solubilize peptidoglycan and eliminate the toga. Acetone was subsequently added to the supernatant. However, in our hands, the digested peptidoglycan material remained soluble, contrary to the observation of Huber et al. (7) Nevertheless, its amino acid and hexosamine analysis was similar to the one reported by these authors, Apm being absent and MurNAc, GlcNAc, Ala, Glu, and Lys being found in the 0.9:1.0:1.6:1.1:1.0 ratio. To determine the stereochemistry of the amino acids, the acid hydrolysate was derivatized with Marfey’s reagent (15). Only Glu and Ala could be analyzed by this method, because the derivatives of L- and D-Lys co-eluted with the huge reagent peak. The configuration of lysine residues was alternatively determined by incubating the hydrolysate with L-Lys decarboxylase (12). The proportions found for the enantiomeric amino acids were as follows: Glu, 2% l and 98% d; Ala, 37% l and 63% d; and Lys, 60% l and 40% d. Here again, these results confirmed those of Huber et al. (7), including the presence of both enantiomers of Lys in roughly equal quantities.

Muropeptides produced by digestion of peptidoglycan with mutanolysin were reduced with sodium borohydride and separated by reversed-phase HPLC. The profile obtained (Fig. 1) was relatively simple, showing four main peaks and a few smaller ones. The amino acid/hexosamine composition of each peak was determined, and the molecular mass was deduced from MALDI-TOF mass spectra in the positive and negative modes (Table 1). Molecular masses suggested that peaks I, II, and VII were monomers while peaks III, IV, V, VI, VIII, and IX were dimers.

Structure of the Main Muropeptides—The structure of the muropeptides in peaks I–IV was determined by a combination of several approaches (Table 1). Analyses with L-Lys decarboxylase allowed the determination of the stereochemistry of lysine in each muropeptide. The quantification of the isomers of alanine was performed with d-amino acid oxidase. In our assay conditions, the enzyme efficiently oxidized d-Ala (98%) but not d-Glu and d-Lys (10%). The acylation status of the α- and ε-amino groups of lysine residues was determined by dinitrophenylation (12). Owing to the small amount of material available, a HPLC procedure for the separation of the dinitrophenyl derivatives was set up (Fig. 2), which proved to be more sensitive than the usual TLC procedure (12).

Muropeptide I was found to contain one residue each of L-Ala, d-Glu, and d-Lys (Table 1). The dinitrophenylation assay showed that the α-amino group of d-Lys was free, whereas the ε-amino group was acylated (Fig. 2). Thus, muropeptide I was a tripeptide monomer in which d-Lys was acylated by d-Glu on its ε-amino function (l-AlaI–d-GluII(γ→ε)–d-Lys); Fig. 3). The calculated monoisotopic mass of the disaccharide-tripeptide matched the m/z values obtained by MALDI-TOF analyses in the positive and negative modes (Table 1).

Muropeptide II differed from I by the presence of an additional d-Ala, of l-Lys instead of d-Lys, and of a free ε-amino on the latter residue (Table 1 and Fig. 2). Thus, d-Glu was linked to l-Lys by the classic γ→α amide bond (3). The inferred struc-
Table 1

| Muropeptide | Retention time | Relative abundancea | Murb | Gluc | Alac | Glcnb | Lysd | α-Lys contenta | ε-Ala contenta | MALDI-TOF m/z | Calculated monoisotopic molecular mass |
|-------------|----------------|---------------------|------|------|------|-------|------|----------------|----------------|------------|-------------------------------------|
| I           | 37.5           | 26.2                | 0    | 1.2  | 0.9  | 0.9   | 1    | 98             | 91             | 826.9      | 825.1                 | 826.4               |
| II          | 39.5           | 50.8                | 0    | 1.2  | 2.0  | 0.9   | 1    | 4              | 51             | 1378.2     | 1376.5                 | 1377.6              |
| III         | 45.9           | 6.9                 | 0    | 1.1  | 2.1  | 1.7   | 1    | 97             | 100            | 1706.2     | 1704.6                 | 1705.5              |
| IV          | 49.9           | 9.3                 | 0    | 1.2  | 1.5  | 0.9   | 1    | 53             | 70             | 1706.2     | 1704.6                 | 1705.5              |
| V           | 52.0           | 1.4                 | 0.5  | 1.2  | 1.6  | 1.2   | 1    | ND             | ND             | 1338.3     | 1336.6                 | 1337.6              |
| VI          | 53.5           | 1.3                 | 0.5  | 1.2  | 1.9  | 1.4   | 1    | ND             | ND             | 1338.3     | 1336.5                 | 1337.6              |
| VII         | 55.1           | 1.0                 | 0.7  | 1.2  | 1.8  | 0.8   | 1    | ND             | ND             | 878.1      | 876.3                  | 877.4               |
| VIII        | 56.1           | 0.9                 | 0.3  | 1.2  | 1.4  | 0.8   | 1    | ND             | ND             | 1686.6     | 1684.9                 | 1685.5              |
| IX          | 56.5           | 1.1                 | 0.4  | 1.2  | 1.6  | 0.9   | 1    | ND             | ND             | 1686.5     | 1684.8                 | 1685.5              |

a Numbers correspond to peaks in Fig. 1.
b From the integration of the absorption areas of peaks eluted between 36 and 60 min.
c With Lys taken as 1.
d Lysine yield, determined with the amino acid analyzer, after reaction of an acid hydrolysate with L-lysine decarboxylase.

A 350 nm

FIGURE 3. Structures of muropeptides I–IV. Arrows represent the direction of peptide or amide bonds from CO to NH. In dimers, the cross-linking bond is represented by an open arrow. Greek letters indicate the carboxyl group of Glu or the amino group of Lys engaged in peptide bonds. MurOHNAc, N-acetylglucosamine (resulting from the reduction of the MurNAc residue).

Structure of T. maritima Peptidoglycan

The amino acid composition of muropeptide IV suggested the presence of a dimer comprising a tetrapeptide cross-linked to a tripeptide. This dimer contained one D-Lys and one ε-Lys. The ε-amino function of one of these lysine residues was free, whereas the two amino functions were substituted in the other. These results indicated that muropeptide IV contained a tetrapeptide stem (L-Ala-D-Glu-L-Lys-D-Ala) connected to a tripeptide stem (L-Ala-D-Glu-ε-Lys3) by a D-Ala(α→ε)-Lys3 cross-link (Fig. 3), a structure that was confirmed by tandem mass spectrometry (supplemental Fig. S1). Thus, the peptide stems of dimer IV displayed the sequences of monomers I and II in the acceptor and donor positions, respectively.
Structure of T. maritima Peptidoglycan

Structure of the Minor Muropeptides—Owing to the limited amount of material collected, amino acid analyses of minor peaks V–IX (Table 1) were not as accurate as those of the main peaks. Nevertheless, the structure of these muropeptides could be inferred from two characteristics: contrary to the main peaks, in which MurNAc had been converted into N-acetylmyramicinol after reduction (16), they released muramic acid upon acid hydrolysis, and they displayed a 20-Da mass defect with respect to peaks II, III, and IV. Therefore, they corresponded to anhydro derivatives of the latter compounds (19, 20).

Cross-linking Index and Average Chain Length of the Glycan Chains—From the integration of the peaks of the HPLC profile (Fig. 1 and Table 1), two parameters of T. maritima peptidoglycan, the cross-linking index and the average chain length, were estimated. The cross-linking index, defined as the percentage of cross-links relative to the total number of disaccharide-peptide subunits (16), was 10. The average chain length, calculated from the percentage of 1,6-anhydro disaccharide peptides (16), was 30 disaccharide units.

DISCUSSION

In Gram-negative organisms, the amino acid at position 3 of the peptide stem is generally meso-A3pm with a few exceptions, such as L-ornithine in spirochetes (2, 3). Therefore, the occurrence of lysine at this position in the peptidoglycan of T. maritima was surprising. Even more surprising was the co-existence of both enantiomers of lysine, the D-isomer representing as much as 40% of the overall peptidoglycan content of this residue. D-Lys has only been previously found in the peptidoglycan of Butyribacterium rettgeri where it participates in the peptide cross-bridge (21). To the best of our knowledge, the current analysis provides the first direct determination of the position of D-Lys in the peptide stems of peptidoglycan.

Two different amino acids at position 3 of stem peptides have been detected in Bifidobacterium globosum (L-Lys and L-Orn) (22) and Myxococcus xanthus (meso-A3pm and LL-A3pm) (23). This results from the unusually low specificity of the MurE ligase of these species (14). In T. maritima, MurE catalyzes in vitro two modes of acylation of the lysine enantiomers: L-Lys is conventionally acylated on its α-amino function, whereas D-Lys is acylated on its ε-amino function. This leads to the unusual γ→ε arrangement of the D-Glu2-D-Lys3 amide bond. We show here that these two modes of acylation operate in vivo, leading to formation of monomers I and II in similar amounts. D-Lys was only detected in the tripeptide monomer (I), reflecting the impossibility for the MurF ligase to add D-Ala-D-Ala to the D-Lys-containing nucleotide precursor (12).

Strikingly, structural analysis of T. maritima peptidoglycan revealed that D-Lys participated in two novel types of cross-linking. The first cross-link, present in dimer IV, originates from a transpeptidation reaction involving a conventional L-Lys-containing pentapeptide donor and a D-Lys-containing tripeptide acceptor. In the resulting 4→3 cross-link, the α-amino group of D-Lys plays the role of the side-chain amino groups of meso-A3pm found in most Gram-negative bacteria. Thus, dimer IV is likely to be cross-linked by classic DD-transeptidases belonging to the PBP family. Indeed, this class of active-site serine enzymes functions with conserved donors ending in D-Ala-D-Ala, or rarely in D-Ala-D-Ser and D-Ala-D-Ser lactate, whereas extensive variation is tolerated in the residue that carries the reacting amino group in the acceptor (17). Impaired growth of T. maritima in the presence of penicillin (7) is in agreement with this notion. None of the dimers detected in the peptidoglycan of T. maritima contained 4→3 cross-links involving L-Lys at position 3 in the acceptor stem. This observation suggests that the DD-transeptidases of this species are specific of amino groups that are linked to a carbon bearing also a carboxyl group and having the D configuration. The DD-transeptidases of Escherichia coli display the same specificity since incorporation of L-Lys instead of meso-A3pm into peptidoglycan results in cell lysis (24). Thus, D-Lys appears to act as a surrogate of meso-A3pm by providing the essential amino group and its accompanying carboxyl group with the D configuration of the asymmetric carbon. One major consequence of this model is that the donor and the acceptor substrates of the transpeptidation reaction have distinct structures, an L-Lys-containing pentapeptide ending in D-Ala-D-Ala and a D-Lys-containing tripeptide. The control of the supply of the two types of peptide stems to the DD-transeptidases is therefore likely to be critical for peptidoglycan polymerization and cell wall morphology.

The second cross-link, present in dimer III, originates from the L-Ala1-D-Glu2 bond of a peptide stem as the acyl donor and the D-Lys-containing tripeptide as the acceptor, generating a 1→3 cross-link. To date, four modes of peptidoglycan cross-linking have been observed in the bacterial world (3). The most frequent 4→3 cross-links produced by active-site serine PBPs are present in T. maritima as discussed above. In Micrococcus luteus, 4→1 cross-links involve “head-to-tail” transpeptidation of the peptide stem (25). In this case, the amino group of L-Ala at position 1 of the acceptor stem is thought to be generated by cleavage of the MurNAc-L-Ala3 amide bond. In certain coryneform bacteria, 4→2 cross-links have been found (2). This par-
ticular mode of cross-linking requires a diamino acid between D-Ala at position 4 and D-Glu at position 2. The latter modes of cross-linking (4→1 and 4→2) should involve as yet uncharacterized DD-transpeptidases that cleave the D-Ala¹-D-Ala¹ peptide bond of the donor and use different amino groups in the acceptor. In contrast, the remaining cross-links of the 3→3 mode are produced by active-site cysteine LD-transpeptidases that cleave the L-Lys²-D-Ala³ peptide bond of the donor in β-lactam-resistant E. faecium and non-replicative forms of M. tuberculosis (see the introduction) (5, 6). The 1→3 cross-link found in the present work is a novel mode of cross-linking. The nature of the enzyme catalyzing its formation is unknown. In Bacillus subtilis, an enzyme (LytH) hydrolyzes the L-Ala¹-D-Glu² bond of the polymer during endospore cortex formation (26). A gene homologous to lytH is present in the T. maritima genome (blast.ncbi.nlm.nih.gov/Blast.cgi). By analogy with active-site serine enzymes belonging to the PBP family, that comprise both hydrolases (DD-carboxypeptidases) and biosynthetic enzymes (DD-transpeptidases), the LytH homologue of T. maritima could generate the 1→3 cross-links by functioning as a transpeptidase rather than a hydrolase. The absence of disaccharide-L-Ala in the T. maritima peptidoglycan, contrary to that of B. subtilis endospores (27), suggests an exclusive biosynthetic role for the putative transpeptidase.

The structural analysis of T. maritima peptidoglycan reveals additional features. The existence of anhydro-muropeptides V–IX shows that the glycan strands do not terminate with a reducing MurNAc residue but with 1,6-anhydro-MurNAc, as is the case in many bacteria (28). The absence of detectable L-Lys-containing pentapeptide or tripeptide should also be noted. This means that (i) the DD-carboxypeptidases have removed most (if not all) of the D-Ala residues at position 5 remaining after transpeptidation, and (ii) no LD-carboxypeptidase or LD-endopeptidase activity is present, or at least active, during the normal growth of T. maritima.

T. maritima peptidoglycan appears to be loosely cross-linked as indicated by the cross-linking index estimated here (10%), a value that is low when compared with that of E. coli (25%) (16, 29) or other Gram-negative bacteria (26–60%) (30). On the other hand, the average chain length found (30 disaccharide units) is comparable to that of E. coli (26–38 disaccharide units) (16, 29). In other Gram-negative species, this parameter varies widely, from 10 to 53 disaccharide units (30). Therefore, the glycan strands in T. maritima are fairly long.

Hyperthermophiles such as thermotogales are adapted to hot environments by their physiological and nutritional requirements (31). As a consequence, cell components such as lipids, nucleic acids, and proteins, which are usually heat-sensitive, have to be stable at temperatures as high as 70–90°C (32). For instance, membrane lipids of T. maritima contain a glycerol ether lipid which, in contrast to ester lipids found in mesophiles, may increase the stability of membranes against hydrolysis at high temperature (33). Whether the unusual 1→3 cross-links discovered in this study contribute to the thermostability of the cell wall of T. maritima is unknown. Such a contribution might relate to the constraint imposed by 1→3 cross-links to the distance separating adjacent glycan chains: indeed, two cross-linked disaccharide subunits are connected by 4 amino acid residues in this case, instead of 7 and 12 in E. coli and S. aureus peptidoglycans, respectively. However, further experiments will be necessary to ascertain that this structural feature is linked to the thermophilic character of T. maritima.

In conclusion, we have investigated here the composition and fine structure of T. maritima peptidoglycan. Analyses have revealed for the first time the key role of D-Lys in peptidoglycan synthesis, both as a surrogate of meso-A,pm at the third position of peptide stems, and in the formation of two novel types of cross-link, namely L-Ala¹(α→α)d-Lys³ and D-Ala⁴(α→α)d-Lys³.

Acknowledgment—T. maritima cells were kindly provided by Dr. Claire Bouthier de la Tour.

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Structure of T. maritima Peptidoglycan

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