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# Cell wall structural divergence among *Thermus* spp.

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**Abstract**

The fine structure of sacculi from *Thermus thermophilus* HB27, *T. aquaticus* YT-1 and *Thermus* ATCC27737 has been worked out by HPLC analysis and mass spectrometry techniques. The three microorganisms have a murein composition of the rare A3β chemotype, but showed substantial differences in muropeptide composition. Phenylacetylated muropeptides, previously described in *T. thermophilus* HB8, were detected exclusively in *T. thermophilus* HB27. Murein from *T. aquaticus* YT-1 was devoid of D-Ala-D-Ala terminated muropeptides, which were, in contrast, abundant in *T. thermophilus* HB27 and *Thermus* ATCC27737. The significance of these findings is discussed. 

*Keywords:* Peptidoglycan; Murein; Cell wall; Mass spectrometry; *Thermus*

## 1. Introduction

The peptidoglycan (murein) sacculus constitutes the primary morphogenetic element of the bacterial cell [1]. Although murein basic architecture is highly conserved among bacteria, a large number of variations have been identified [2,3]. Variations in murein structure likely reflect evolutionary adaptations to particular environments, and have important taxonomic implications [3].

*Thermus* spp. are extreme thermophilic bacteria living in hot springs [4]. Together with the genus *Deinococcus* form one of the earlier branches in bacterial phylogenetic trees [5]. Investigation of *Thermus thermophilus* HB8 cell wall revealed a unique murein structure [6]. First, the primary structure (monomeric subunit: *N*-acetylglucosamine-*N*-acetylmuramyl-L-Ala-D-Glu-(γ)-L-Ornithinyl [(δ)Gly-Gly]-D-Ala-D-Ala) corresponded to the rare A3β chemotype only found before in the phylogenetically related species *Deinococcus radiodurans* [3,7]. Second, about 25% of total muropeptides have the (δ)$NH_2$ of ornithine (Orn) acylated by phenylacetyl-Gly instead of $(Gly)_2$. *T. thermophilus* HB8 is the only known case with phenylacetate (PA) as a murein component. The extreme rarity of *T. thermophilus* HB8 murein structure, led us to investigate whether or not its distinctive features were conserved among other members of the genus, as they could represent

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important taxonomical traits for the genus *Thermus*. Therefore murein from representative strains of the species *T. thermophilus* and *T. aquaticus*, and from the isolate *Thermus* ATCC27737 was investigated.

## 2. Materials and methods

### 2.1. Bacteria and growth conditions

*T. thermophilus* HB27 [8], *T. aquaticus* YT-1 (ATCC25104) and *Thermus* sp. ATCC27737 were used in this work. Bacteria were grown at 70°C in 4 g $l^{-1}$ yeast extract, 8 g $l^{-1}$ Bacto Peptone, and 3 g $l^{-1}$ of NaCl, pH 7.5 [9], except *T. aquaticus* YT-1 which was grown in the same medium diluted to one half.

### 2.2. Murein preparation and HPLC analyses

Bacterial suspensions were dropped onto an equal volume of boiling 8% sodium dodecylsulfate and kept for 1 h under magnetic stirring. Detergent was removed by repeated cycles of centrifugation and resuspension in water. Murein samples were muramidase (Cellosyl, Hoechst, Germany) digested and analysed by HPLC on a Hypersil ODS 18 reverse-phase column (250×4 mm, 3 mm particle size) (Teknochroma, Barcelona, Spain) [6,10,11]. Muropeptides were detected by monitoring $A_{204}$. For amino acid analysis murein samples were hydrolysed in 6 N HCl for 12 h at 105°C, vacuum dried, resuspended in water, and further processed for *ortho*-phtaldialdehide pre-column derivatisation and HPLC analysis as described [6].

### 2.3. HPLC analysis of muropeptide-derived lactylpeptides

Samples (500 μg) of unreduced muropeptide mixtures were subjected to β-elimination with 4 M $[NH_4]OH$ for 1 h at 37°C [12]. The mixtures were vacuum dried, resuspended in water, and analysed by HPLC on a Hypersil RP 18 column (3 μm of particle size, 250×4 mm) at room temperature. Column was eluted at 0.5 ml $min^{-1}$ for 5 min with 0.05% trifluoracetic acid (TFA) in ultrapure water (MilliQ system, Millipore), and then with a 115 min linear gradient to 30% acetonitrile, 0.05% TFA in ultrapure water. Eluent was monitored on an UV

Table 1
Calculated and measured *m/z* values for the positive quasimolecular ions of major lactylpeptides

| Lactylpeptide[c] (Peak in Fig. 1A)[d] | Ion type | *T. aquaticus* YT-1 | | | | *Thermus* ATCC27737 | | |
|---|---|---|---|---|---|---|---|---|
| | | Calculated *m/z* | Measured *m/z* | Δm[a] | Error (%)[b] | Measured *m/z* | Δm[a] | Error (%)[b] |
| Gly-(δ)-L-Orn[(α)-**R**]-D-Ala-Gly+Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala (4+5) | $[M+Na]^+$ | 612.60 | 612.74 | +0.14 | +0.02 | 612.74 | +0.14 | +0.02 |
| | $[M+K]^+$ | 628.71 | 628.86 | +0.15 | +0.02 | 628.86 | +0.15 | +0.02 |
| | $[M+Na+K-H]^+$ | 650.69 | 650.82 | +0.13 | +0.02 | 650.82 | +0.13 | +0.02 |
| | $[M+2Na+K-2H]^+$ | 672.68 | 673.11 | +0.43 | +0.06 | n.d. | – | – |
| Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala → Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala (10) | $[M+Na]^+$ | 1184.19 | 1184.73 | +0.54 | +0.05 | 1184.73 | +0.54 | +0.05 |
| | $[M+K]^+$ | 1200.29 | 1201.03 | +0.74 | +0.06 | 1201.30 | +1.01 | +0.08 |
| | $[M+2Na-H]^+$ | 1206.16 | 1206.32 | +0.16 | +0.01 | 1206.32 | +0.16 | +0.01 |
| | $[M+Na+K-H]^+$ | 1222.27 | 1222.77 | +0.50 | +0.04 | 1222.72 | +0.50 | +0.04 |
| | $[M+2Na+K-2H]^+$ | 1244.26 | n.d. | – | – | 1242.02 | −2.24 | −0.18 |
| Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala → Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala → Gly-Gly-(δ)-L-Orn[(α)-**R**]-D-Ala (13) | $[M+Na]^+$ | 1755.76 | 1756.65 | +0.89 | +0.05 | 1756.65 | +0.89 | +0.05 |
| | $[M+K]^+$ | 1771.87 | 1772.64 | +0.74 | +0.04 | 1772.64 | +0.77 | +0.04 |
| | $[M+Na+K-H]^+$ | 1793.85 | 1795.80 | +1.95 | +0.11 | n.d. | – | – |
| | $[M+2Na+K-2H]^+$ | 1815.84 | n.d. | – | – | 1813.91 | −1.93 | −0.11 |

[a]Mass difference between measured and calculated quasimolecular ion values.
[b][(measured mass−calculated mass)/calculated mass]×100.
[c]R, D-lactyl-L-Ala-D-Glu-(γ)→.
[d]Source muropeptides in Fig. 1A for the indicated lactylpeptides.

detector set at 204 or 258 nm. Desalting of lactylpeptide mixtures was performed by HPLC as above except for the elution program: 8 min 0.05% TFA in water, 20 min linear gradient to 30% acetonitrile, 0.05% TFA and 15 min isocratic in 30% acetonitrile, 0.05% TFA. Lactylpeptides eluted between 18 and 32 min and were collected as one fraction (7 ml) which was lyophilised and subjected to further analysis.

### 2.4. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry

Measurements were performed on a Kompact MALDI III (Shimadzu Kratos Analytical, Manchester, UK) laser desorption time-of-flight instrument equipped with a type VSL-337ND (Laser Science, Newton, MA, USA) nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width). Positive ions were accelerated from the target in the continuous mode to a final potential of 20 kV and laser power density was kept near threshold level. All spectra were acquired in the reflector mode. Mass spectra were obtained by signal averaging of 100 laser shots and then smoothed by Savitsky-Golay algorithm. Calibration of the instrument was done externally with the $[M+H]^+$ ions of the applied matrix and the $[M+H]^+$ ions of standard peptides (Leu-Trp-Met-Arg-Phe-Ala, angiotensin III, substance P and bee venom mellitin). Average mass values are reported. Sample preparation was as reported in [13] with α-cyano-4-hydroxy-*trans*-cinnamic acid matrix (Sigma, St. Louis, MO, USA). 0.5 μl of the matrix solution (20 mg $ml^{-1}$ acetone) was placed in the centre of each sample well of the disposable stainless-steel 20-well target and the solvent was evaporated quickly at room temperature. The desalted mixture of lactylpeptides was redissolved in 50 μl of water containing 0.1% TFA. Sample solution (0.5 μl) was placed on top of the matrix surface, dried and inserted into the ion source.

## 3. Results

### 3.1. Muropeptide composition of murein from Thermus spp.

Amino acid analysis of muramidase (Cellosyl

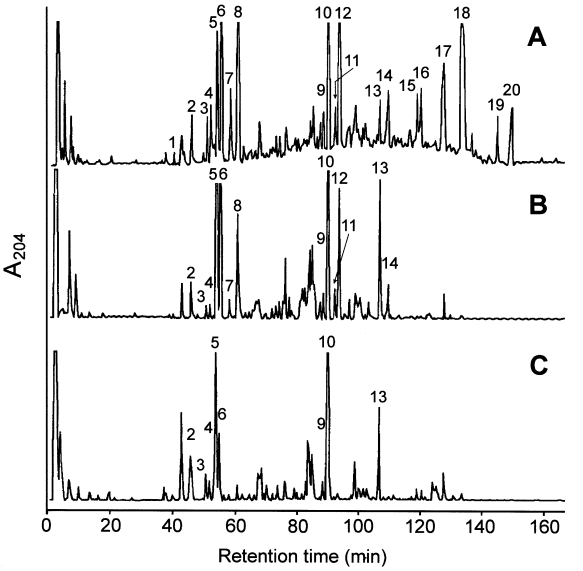


Fig. 1. HPLC analysis of murein samples. Murein from (A) *T. thermophilus* HB27, (B) *Thermus* ATCC27737 and (C) *T. aquaticus* YT-1 were subjected to HPLC analysis. Numbers in the chromatogram for *T. thermophilus* HB27 correspond to: 1, L-Orn[(α)-R]-D-Ala; 2, $(Gly)_2$-(δ)-L-Orn[(α)-R]-Gly; 3, L-Orn[(α)-R]-$(D\text{-}Ala)_2$+Gly-(δ)-L-Orn[(α)-R]-D-Ala; 4, Gly-(δ)-L-Orn[(α)-R]-D-Ala-Gly; 5, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala; 6, $(Gly)_2$-(δ)-L-Orn-[(α)-R]-D-Ala-Gly; 7, Gly-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 8, $(Gly)_2$-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 9, Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala; 10, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala+$(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala-Gly; 11, Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 12, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 13, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala; 14, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 15, **PA**-Gly-(δ)-L-Orn[(α)-R]-Gly+ Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-(Anh)R]-$(D\text{-}Ala)_2$; 16, $(Gly)_2$-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-(Anh)R]-$(D\text{-}Ala)_2$; 17, **PA**-Gly-(δ)-L-Orn[(α)-R]-D-Ala+**PA**-Gly-(δ)-L-Orn[(α)-R]-D-Ala-Gly; 18, **PA**-Gly-(δ)-L-Orn[(α)-R]-$(D\text{-}Ala)_2$; 19, **PA**-Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-(Anh)R]-D-Ala+**PA**-Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-(Anh)R]-D-Ala-Gly; 20, **PA**-Gly-(δ)-L-Orn[(α)-R]-D-Ala → $(Gly)_2$-(δ)-L-Orn[(α)-(Anh)R]-$(D\text{-}Ala)_2$. Abbreviations: R, *N*-acetylglucosaminyl-(β 1 → 4)-*N*-acetylmuramyl-L-Ala-D-Glu-(γ) → ; (Anh)R, *N*-acetylglucosaminyl-(β 1 → 4)-[(1 → 6) anhydro]-*N*-acetylmuramyl-L-Ala-D-Glu-(γ) → ; **PA**, phenylacetate. Numbers in the chromatograms for *Thermus* ATCC27737 and *T. aquaticus* YT-1, identify the corresponding muropeptides with respect to *T. thermophilus* HB27, assuming identity of peaks with equal retention times.

Hoechst, Frankfurt am Main, Germany) digested sacculi from *T. thermophilus* HB27, *Thermus* ATCC27737, and *T. aquaticus* YT-1 revealed Glu, Orn, Ala and Gly as the only amino acids in the muramidase-solubilised material. In all instances less than 3% of the total amount of Orn, remained insoluble after muramidase treatment, indicating a satisfactory solubilisation of the mureins.

Murein samples (200 μg) were muramidase digested, and the solubilised fractions were analysed by HPLC. The corresponding chromatograms are shown in Fig. 1. The elution profile for *T. thermophilus* HB27 was virtually identical to the one established previously for the HB8 strain, including PA containing muropeptides [6]. Coincidence in the retention times (Rt's) of peaks was confirmed running a sample of *T. thermophilus* HB8 murein as reference (data not shown). Therefore peaks with the same Rt's in *T. thermophilus* HB27 and HB8 were considered identical (Fig. 1). Elution profiles for *T. aquaticus* YT-1, and *Thermus* ATCC27737 were quite different to *T. thermophilus*. The more distinctive features were the absence of PA-muropeptides in *T. aquaticus* YT-1 and *Thermus* ATCC27737, and the reduction in the number of major peaks in *T. aquaticus* YT-1 (Fig. 1). Absence of PA-muropeptides was confirmed in HPLC runs with the detector set at 258 nm, where PA but not normal muropeptides absorb strongly. All the significant peaks of *T. aquaticus* YT-1, and *Thermus* ATCC27737 eluted with Rt's coincident with muropeptides of *T. thermophilus*. Lactylpeptide HPLC elution profiles were specific for each strain, although peaks in the simpler chromatograms had equivalents with identical Rt's into the more complex ones (Fig. 2). Such results suggested that mureins from *T. aquaticus* YT-1, and *Thermus* ATCC27737 were made up of subsets of *T. thermophilus* muropeptides. PA-acylated lactylpeptides, identified for their absorption at 258 nm, were found only in *T. thermophilus* HB27 (Fig. 2).

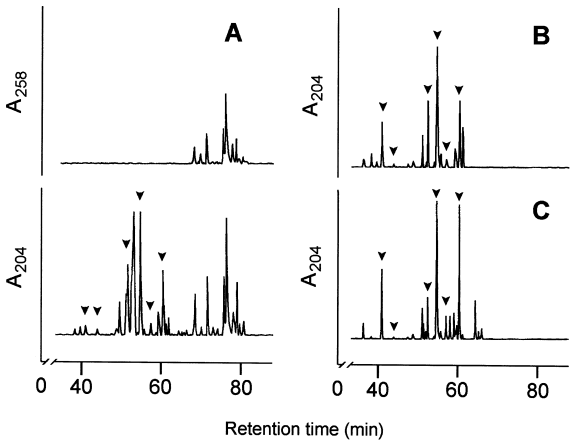


Fig. 2. HPLC analysis of muropeptide-derived lactylpeptides. Muropeptide mixtures from (A) *T. thermophilus* HB27, (B) *Thermus* ATCC27737 and (C) *T. aquaticus* YT-1 were subjected to β-elimination and analysed by HPLC. Eluate was monitored either at 204 or at 258 nm in all instances. Samples B and C did not contain muropeptides detectable at 258 nm. Arrowheads indicate the lactylpeptides common to all samples. Range for *Y*-axis was the same at 204 and 258 nm.

### 3.2. MALDI-TOF mass spectrometry of lactylpeptide mixtures

Mixtures of lactylpeptides from *T. aquaticus* YT-1 and *Thermus* ATCC27737 were analysed by MALDI-TOF to identify individual molecular species present in the mixtures. Sodium-carrying molecular ions of the major lactylpeptides were detected as dominating ions, although more complex sodium and/or potassium adduct ions —— $[M+K]^+$, $[M+Na+K-H]^+$, $[M+2Na+K-2H]^+$ —— were also formed (Fig. 3). The *m/z* values measured for the different quasimolecular ions were compared in Table 1 with values calculated for lactylpeptides derived from muropeptides of *T. thermophilus* [6]. Three identical lactylpeptides were identified in both species (Table 1) which corresponded to the peptide moieties of muropeptides 4 plus 5, 10a and 13 of *T. thermophilus* (Fig. 1) [6]. These results supported a common amino acid sequence for the peptide side chains of mureins from *T. aquaticus* YT-1, *Thermus* ATCC27737 and *T. thermophilus*, and indicated that muropeptides with equal Rt's in the HPLC elution patterns were indeed the same for the three microorganisms.

### 3.3. Muropeptide proportions of Thermus spp. mureins

Muropeptide proportions were calculated on the assumption that HPLC peaks with equal Rt's correspond to identical muropeptides in all samples. An

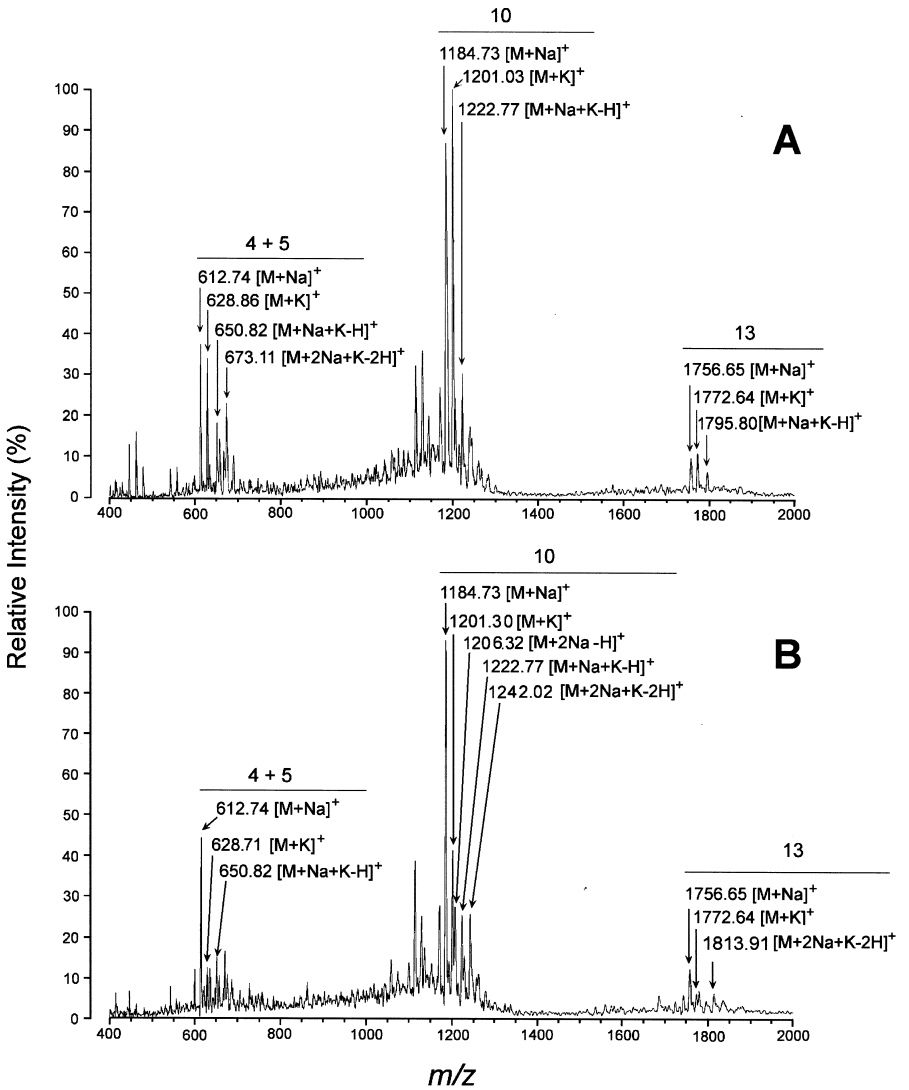


Fig. 3. MALDI-MS analysis of lactylpeptides from (A) *T. aquaticus* YT-1, and (B) *Thermus* ATCC27737. Aliquots of the lactylpeptide mixtures used for HPLC analysis were subjected to MALDI-MS. The series of peaks for each single lactylpeptide are grouped by lines. The numbers indicate the peaks in Fig. 1 able to release the corresponding lactylpeptide.

automatic peak-integrator (Spectra Physics 4270, Spectra-Physics, San Jose, CA, USA) was used and integration areas were corrected for the extinction coefficient of individual muropeptides as stated by Glauner [11], and Quintela [6]. The results are summarised in Table 2. More remarkable features were the apparent lack of muropeptides with D-Ala-D-Ala C-terminal dipeptides in *T. aquaticus* YT-1 (peaks 7, 8, 11, 12 in Fig. 1) and the presence of PA-muropeptides in *T. thermophilus* HB27 only. In all instances dimers were the major cross-linked muropeptides with sizable proportions of trimers but no higher order oligomers were detected.

## 4. Discussion

Muropeptide analysis of murein from *T. aquaticus*

Table 2
Muropeptide composition of murein from *Thermus* spp.

| Muropeptide[a] | Relative abundance (mol %) | | |
|---|---|---|---|
| | *T. thermophilus* HB27 | *Thermus* ATCC27737 | *T. aquaticus* YT-1 |
| 1 | 0.71 | n.d. | n.d. |
| 2 | 3.94 | 3.89 | 6.49 |
| 3[b] | 3.44 | 2.50 | 5.99 |
| 4 | 3.82 | 2.32 | 2.13 |
| 5 | 8.74 | 20.89 | 36.28 |
| 6 | 14.03 | 23.02 | 13.90 |
| 7 | 4.86 | 2.50 | n.d. |
| 8 | 15.61 | 11.34 | n.d. |
| 9 | 2.48 | 2.00 | 2.31 |
| 10[b] | 6.14 | 19.27 | 24.74 |
| 11 | 1.91 | 1.78 | n.d. |
| 12 | 4.86 | 5.14 | n.d. |
| 13 | 1.35 | 3.95 | 8.17 |
| 14 | 0.64 | 1.41 | n.d. |
| 15[b] | 2.87 | n.d. | n.d. |
| 16 | 2.34 | n.d. | n.d. |
| 17[b] | 8.07 | n.d. | n.d. |
| 18 | 10.18 | n.d. | n.d. |
| 19[b] | 2.12 | n.d. | n.d. |
| 20 | 1.89 | n.d. | n.d. |
| Monomers | 74.34 | 66.45 | 64.79 |
| Dimers | 23.67 | 28.19 | 27.04 |
| Trimers | 1.99 | 5.36 | 8.17 |
| D-Ala-D-Ala[c] | 50.43 | 22.97 | n.d. |
| D-Ala-Gly[d] | 25.85 | 34.34 | 28.03 |
| PA[e] | 23.26 | n.d. | n.d. |
| Cross-linkage | 31.6 | 39.0 | 43.39 |

[a]Numbers correspond to peaks in Fig. 1.
[b]Two muropeptides coelute (see legend to Fig. 1).
[c]Muropeptides with D-Ala-D-Ala at the C-terminal of the peptide side chain. As no other D-Ala-D-Ala terminated muropeptide was found in *T. aquaticus* YT-1, it was assumed that the D-Ala-D-Ala muropeptide eluting in peak 3 was also absent.
[d]Muropeptides with D-Ala-Gly at the C-terminal of the peptide side chain.
[e]Muropeptides with phenylacetyl-Gly bound to the ornithine δ-$NH_2$ group.
n.d., not detected.

YT-1, *Thermus* ATCC27737, and *T. thermophilus* HB27 (found to be virtually identical to strain HB8 [6]) demonstrated a substantial diversity in muropeptide composition. Nevertheless, HPLC results were congruent with a common murein basic architecture for the three *Thermus* spp. This was confirmed by the positive identification of identical muropeptides by MALDI-TOF mass spectrometry of lactylpeptide mixtures. Therefore it was concluded that the three mureins conformed to the rare A3β chemotype, as *T. thermophilus* HB8, with *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-ala-D-Glu-(γ)-L-Orn[(δ)-Gly-Gly]-D-Ala-D-Ala as the basic monomer, and $(Gly)_2$ mediated cross-linking [3,6].

Muropeptide compositions were largely different because of variability in the proportion of D-Ala-D-Ala terminated muropeptides, and because PA-muropeptides were only present in *T. thermophilus*. D-Ala-D-Ala muropeptides accounted for about 50% of the total in *T. thermophilus*, but could not be detected in *T. aquaticus* YT-1. The disparity observed could reflect a deficiency in DD-carboxypeptidase activity in *T. thermophilus* as demonstrated for *Caulobacter crescentus* [14]. In contrast, D-Ala-Gly muropeptides were abundant in all the mureins tested (25 to 34 mol %). D-Ala-Gly muropeptides have been found as trace components in *Escherichia coli* [8], and in higher proportions in *C. crescentus* and *D. radiodurans* [7,14]. In *E. coli* D-Ala-Gly muropeptides are generated by D-Ala-D-Ala ligase miss-incorporation of Gly [10,15]. Whether the same mechanism applies to bacteria rich in such muropeptides is not known. Interestingly *T. aquaticus* YT-1 murein was devoid of D-Ala-D-Ala muropeptides but was rich in D-Ala-Gly muropeptides, indicating that the corresponding DD-carboxypeptidase(s) might be highly specific for D-Ala-D-Ala muropeptides.

According to our results accumulation of PA-muropeptides is a distinct characteristic of *T. thermophilus*. The fact that *T. thermophilus* murein had the higher proportion of D-Ala-D-Ala muropeptides but the lower cross-linkage, suggests that PA-muropeptides, blocked as acceptors for transpeptidation, could modulate cross-linkage in *T. thermophilus*. However the previously proposed implication of PA-muropeptides in thermostability is seriously question by their absence from mureins of *T. aquaticus* and ATCC27737, which have growth temperature ranges similar to *T. thermophilus* [6].

The structural differences observed in *Thermus* spp. cell walls imply deep adaptations in murein metabolism. From an evolutive point of view the unique ability of *T. thermophilus* to accumulate PA-muropeptides is most interesting. Synthesis of PA-muropeptides is likely to require a set of dedicated enzymes which could have been in the common ancestor and conserved only in *T. thermophilus*, or

alternatively could have been gained through *T. thermophilus* speciation. In any case, the divergence in the ability to synthesise PA-muropeptides is likely to correspond to some decisive event in the evolutionary history of the genus *Thermus*.

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