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Amino Acid Sequence of the Threonine-Containing Mureins of Coryneform Bacteria

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In a study of the mureins of coryneform bacteria (*Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Erysipelothrix*), 21 threonine-containing strains were found. In several of the strains the amino acid and amino sugar composition of the murein was muramic acid (Mur), glucosamine ($GlcNH_2$), D-Glu, L-Lys, L-Thr, and Ala in a molar ratio of 1:1:1:1:1:4 or 5, and in several other strains it was Mur, $GlcNH_2$, D-Glu, L-Lys, L-Thr, Ala, and L-Ser in a molar ratio of 1:1:1:1:1:3:1. The amino acid sequence of the mureins was determined by analyzing the oligopeptides derived from partial acid hydrolysates. It was shown that there were five different murein types. The peptide subunits attached to the muramic acid are the same, namely L-Ala-D-GluNH_2-L-Lys-D-Ala. In one strain, the α-carboxyl group of D-Glu is substituted by D-alanine amide. The interpeptide bridges of the different types consist of the peptides L-Ala-L-Thr-L-Ala, L-Ala-L-Thr, L-Ala-L-Ala-L-Thr, L-Ala-L-Ala-L-Ala-L-Thr, or L-Ala-L-Thr-L-Ser which are bound through their C-termini (L-Ala, L-Thr, L-Ser) to the ϵ-amino group of L-Lys of one peptide subunit and by their N-termini (L-Ala) to the C-terminal D-Ala of an adjacent peptide subunit. Determination of the N- and C-terminal groups in the mureins showed that about 15 to 30% of the interpeptide bridges are not cross-linked.

During the last years the structure of the murein (peptidoglycan) has been elucidated (7). As known so far, the amino acid sequence of the murein of gram-negative bacteria is identical, whereas that of the gram-positive bacteria varies considerably. The most variable portion in the primary structure is the interpeptide bridges which mediate the cross-linking of adjacent peptide subunits (7, 18). Glycine, alanine, serine, aspartic acid, glutamic acid, and sometimes threonine may be present in various numbers and sequences.

As known so far, L-threonine has been found at different positions of the interpeptide bridge. In the murein of *Micrococcus roseus*, an L-Ala-L-Ala-L-Ala-L-Thr interpeptide bridge connects two adjacent peptide subunits (14). The N-terminus of the interpeptide bridge (L-Ala) is bound to the C-terminal D-alanine of one peptide subunit, and the C-terminus of the interpeptide bridge (L-Thr) is attached to the ϵ-amino group of L-lysine of another peptide subunit. In the mureins of *Streptococcus* sp. (20) and *Streptococcus bovis* (11), an L-Ala-L-Thr interpeptide bridge was found. A third threonine-containing interpeptide bridge was described for *Bifidobacterium longum*. It is rather complex, with the following amino acid sequence: L-Ala-L-Thr-L-Ala-L-Ser (12).

In studying the mureins of coryneform bacteria we have found 21 strains which also contain threonine in the cell walls combined with alanine and serine as in the known threonine-containing mureins. This paper describes experiments which show that they belong to four different murein types not described so far.

MATERIALS AND METHODS

Organisms and growth conditions. The following strains were used: *Arthrobacter aurescens* ATCC 13344, *A. citreus* ATCC 11624, *A. globiformis* NCIB 8717, *A. histidinolovorans* ATCC 11442, *A. oxydans* ATCC 14358, *A. oxydans* ATCC 14359, *A. polychromogenes* ATCC 15216, *Arthrobacter* sp. NCIB 9423, *Arthrobacter* sp. NCIB 9666, *Arthrobacter* sp. ATCC 19141, and *A. ureafaciens* ATCC 7562; *Brevibacterium album* ATCC 15111, *B. ammoniagenes* ATCC 6871, *B. cerinum* ATCC 15112, *B. helvolum* ATCC 11822, *B. helvolum* ATCC 19239;

Corynebacterium alkanum ATCC 21194, *C. ilicis* ATCC 14264, *C. rathayi* ATCC 13659, *Corynebacterium* sp. ATCC 21188, and coryneform organism NCIB 8180.

All strains are typical coryneform bacteria characterized by the occurrence of irregular rod-shaped cells and a more or less complete rod-sphere transformation typical for *Arthrobacter* (5). The organisms were grown in peptone-yeast glucose broth (1% peptone from casein, 0.5% yeast extract [Cenovis], 0.5% glucose; *p*H 7.2) with aeration at 30 C. They were harvested in the logarithmic growth phase, when only few of coccoid cells were present. Prior to harvesting, the cell suspensions were kept at 100 C for 30 min to inactivate autolytic enzymes.

Preparative and analytical methods. Cell walls were prepared by the usual techniques (19). For further purification, the cell walls were incubated with trypsin (CW-Tryp). A portion of the cell walls was extracted with 10% trichloroacetic acid for 3 days at 4 C (CW-TCA), and in some cases a sample of this material was further extracted by formamide at 170 C for 20 min (CW-FA).

Dinitrophenylation of cell walls and peptides was carried out as described previously (16, 19).

Quantitative analyses of amino acids and amino sugars in hydrolysates of cell walls (4 N HCl, 100 C, 16 hr) were performed by automatic amino acid analyzers (Beckman, Biocal). The correction for destruction during hydrolysis was based on the analysis of a calibration mixture which was hydrolyzed in the same way as the cell walls. The extinction coefficient for muramic acid was calculated.

For the separation of the amino acids, amino sugars, and peptides by paper chromatography on Schleicher and Schüll 2043b papers, the following solvent systems were used: I, isopropanol-acetic acid-water, 75:10:15 (v/v/v); II, α-picoline-NH_4OH-water, 70:2:28 (v/v/v).

Dinitrophenyl (DNP) amino acids were identified by thin-layer chromatography on cellulose MN 300 (Machery and Nagel, Düren) and silica gel plates (Merck) by using the following solvent systems: III, 1.5 M phosphate buffer (*p*H 6.0); IV, *n*-propanol-0.2% ammonia, 80:10 (v/v); V, benzene-acetic acid, 80:20 (v/v).

Peptides from the partial acid hydrolysates were isolated by repeated one-dimensional paper chromatography in solvent systems I and II and also from two-dimensional paper chromatograms.

The identification of the peptides was carried out by determining the amino acid composition, the position on two-dimensional paper chromatograms, the N-terminal amino acid, and the configuration of alanine in the case of alanine-containing peptides. More complex peptides were subjected to partial hydrolysis, and the dipeptides arising were identified.

Hydrazinolysis of cell walls performed with water-free hydrazine for 80 hr at 80 C (4).

The configuration of glutamic acid and lysine was determined by measuring the optical rotatory dispersion of the DNP derivatives (DNP-Glu and bis-DNP-Lys) at 418 nm. The configuration of serine and threonine was tested by using D-amino acid oxidase (12). The quantitative determination of L- and D-alanine was carried out enzymatically (3).

Enzymes and substrates were obtained from Boehringer, Mannheim. Authentic DNP derivatives came from Serva Entwicklungslabor, Heidelberg.

RESULTS

Molar ratios of the amino acids and amino sugars. The amino acids and amino sugars of the mureins were qualitatively and quantitatively determined in hydrolysates (4 N or 6 N HCl, 100 C, 16 hr) of CW-Tryp, CW-TCA, and partly CW-FA. As shown in Table 1, the cell walls of all strains contain, besides the usual amino sugars muramic acid and glucosamine, the amino acids glutamic acid, lysine, alanine, and threonine; several strains revealing additional serine are compiled in Table 2. In all cell wall preparations glutamic acid, lysine, threonine, and serine (if present) occur in equimolar ratios. The smaller amounts of threonine and lysine in the strains no. 8 to 11 (Table 1) are attributed to incomplete hydrolysis. Correspondingly, the relatively stable dipeptide N^6-threonyl-lysine was found in the hydrolysates (6 N HCl, 100 C, 16 hr). Like N^6-aspartyl-lysine it is split completely by hydrolysis with 6 N HCl, 120 C for 18 hr (8, 20, 21).

Alanine occurs in different molar ratios. In the case of the serine-containing cell walls (Table 2) there are approximately 3 moles per mole glutamic acid; in the case of *C. rathayi* ATCC 13659 and *Corynebacterium* sp. ATCC 21188, 5 moles; and in the cell walls of strains no. 1 through 6 and 10 (Table 1), about 4 moles.

In most of the serine-containing strains, glucosamine is present in excess compared to muramic acid (Table 2). The amount drops after trichloroacetic acid extraction, and further treatment with formamide removes, in the case of *A. oxydans* ATCC 14358, about half of the glucosamine. This indicates that the excess glucosamine is not a constituent of the murein but of the polysaccharide of the cell walls. Thus the ratio muramic acid to glucosamine of the murein is 1:1.

In the hydrolysates of all cell walls, more than 1 mole ammonia per mole glutamic acid was found. This finding and the observation that after 1 hr of hydrolysis 1 mole ammonia per mole glutamic acid is split off demonstrate that ammonia is derived from a carboxyl amide (7).

Configuration of amino acids. The configurations of glutamic acid and lysine were determined only for the murein of *A. aurescens* ATCC 13344. Measurement of the optical rotatory dispersion of the DNP-glutamic acid and

TABLE 1. *Quantitative amino acid and amino sugar composition of threonine-containing cell walls of coryneform bacteria*

Strain	CW prepn[a]	Glu	Ala	Thr	Lys	Mur	$GlcNH_2$	NH_3
1. *Arthrobacter aurescens* ATCC 13344	CW-Tryp	0.29[b]	1.11	0.26	0.31	0.28	0.26	0.40
		1.00[c]	3.88	0.90	1.08	0.98	0.90	1.41
	CW-TCA	0.45	1.58	0.39	0.43	0.33	0.38	0.51
		1.00	3.53	0.86	0.97	0.75	0.84	1.13
	CW-FA	0.58	2.22	0.47	0.60	0.52	0.46	0
		1.00	3.85	0.81	1.03	0.90	0.80	0
2. *A. histidinolovorans* ATCC 11442	CW-Tryp	0.31	1.19	0.27	0.33	0.28	0.36	0.42
		1.00	3.82	0.86	1.06	0.91	1.16	1.36
3. *Arthrobacter* sp. ATCC 19141	CW-Tryp	0.31	1.07	0.26	0.31	0.28	0.28	0.47
		1.00	3.47	0.84	1.01	0.89	0.91	1.52
4. *A. ureafaciens* ATCC 7562	CW-Tryp	0.31	1.10	0.27	0.32	0.29	0.31	0.41
		1.00	3.54	0.86	1.03	0.94	1.01	1.33
5. *Brevibacterium helvolum* ATCC 19239	CW-Tryp	0.26	1.00	0.26	0.26	0.23	0.23	0.37
		1.00	3.79	0.97	0.99	0.88	0.87	1.39
6. *Corynebacterium ilicis* ATCC 14264	CW-Tryp	0.32	1.18	0.30	0.32	0.26	0.35	0.41
		1.00	3.73	0.95	1.00	0.83	1.09	1.30
7. *A. citreus* ATCC 11624	CW-Tryp	0.31	1.18	0.24	0.28	0.26	0.32	0.55
		1.00	3.82	0.78	0.90	0.84	1.02	1.79
	CW-TCA	0.67	2.53	0.51	0.51	0.55	0.49	0.70
		1.00	3.77	0.76	0.77	0.82	0.73	1.04
	CW-FA	0.64	2.44	0.49	0.53	0.53	0.44	0
		1.00	3.80	0.76	0.83	0.82	0.69	0
8. *C. rathayi* ATCC 13659	CW-Tryp	0.21	1.04	0.17	0.18	0.18	0.66	0.43
		1.00	4.92	0.78	0.85	0.83	3.12	2.01
		0.47	2.23	0.36	0.38	0.36	0.48	0.61
		1.00	4.72	0.75	0.81	0.76	1.01	1.29
		0.61	2.95	0.51	0.60	0.51	0.39	0
		1.00	4.81	0.83	0.98	0.83	0.64	0
9. *Corynebacterium* sp. ATCC 21188	CW-Tryp	0.23	1.14	0.19	0.19	0.19	0.73	0.45
		1.00	4.91	0.82	0.81	0.83	3.14	1.94
10. *Arthrobacter* sp. NCIB 9423	CW-Tryp	0.43	1.63	0.33	0.35	0.29	0.30	0.69
		1.00	3.78	0.76	0.81	0.66	0.69	1.59
	CW-TCA	0.74	2.87	0.58	0.61	0.52	0.44	0.90
		1.00	3.90	0.79	0.83	0.71	0.60	1.23
	CW-FA	0.66	2.61	0.52	0.57	0.44	0.46	0
		1.00	3.96	0.78	0.86	0.67	0.69	0

[a] Abbreviations: CW-Tryp, cell walls purified by digestion with trypsin; CW-TCA, cell walls additionally extracted with trichloroacetic acid; CW-FA, cell walls extracted with formamide.

[b] The top number of each pair is amount expressed as micromoles per milligram of cell walls.

[c] The bottom number of each pair is amount relative to glutamic acid (Glu = 1).

TABLE 2. *Quantitative amino acid and amino sugar composition of cell walls of coryneform bacteria containing threonine and serine*

Strain	CW prepn[a]	Glu	Ala	Thr	Ser	Lys	Mur	$GlcNH_2$	NH_3
Arthrobacter globiformis NCIB 8717	CW-Tryp	0.29[b]	0.80	0.26	0.26	0.27	0.26	0.65	0.43
		1.00[c]	2.74	0.90	0.88	0.92	0.90	2.22	1.46
A. oxydans ATCC 14358	CW-Tryp	0.28	0.74	0.24	0.25	0.28	0.22	0.79	0.55
		1.00	2.62	0.86	0.88	0.99	0.79	2.83	1.97
	CW-TCA	0.30	0.75	0.29	0.29	0.29	0.24	0.81	0.44
		1.00	2.49	0.98	0.98	0.96	0.81	2.72	1.46
	CW-FA	0.43	1.09	0.40	0.42	0.41	0.32	0.57	0
		1.00	2.53	0.94	0.97	0.95	0.75	1.33	0
A. oxydans ATCC 14359	CW-Tryp	0.28	0.76	0.24	0.24	0.25	0.22	0.81	0.51
		1.00	2.70	0.85	0.84	0.88	0.79	2.89	1.83
A. polychromogenes ATCC 15216	CW-Tryp	0.42	1.27	0.39	0.39	0.41	0.33	0.33	0.55
		1.00	3.00	0.92	0.93	0.96	0.78	0.78	1.31
Arthrobacter sp. NCIB 9666	CW-Tryp	0.22	0.60	0.21	0.20	0.23	0.19	0.40	0.38
		1.00	2.68	0.92	0.89	1.05	0.85	1.81	1.70
Brevibacterium album ATCC 15111	CW-Tryp	0.27	0.77	0.25	0.25	0.25	0.30	0.51	0.36
		1.00	2.90	0.94	0.94	0.94	1.09	1.92	1.35
B. ammoniagenes ATCC 6871	CW-Tryp	0.29	0.82	0.29	0.27	0.28	0.27	0.86	0.44
		1.00	2.83	0.98	0.93	0.97	0.93	2.97	1.50
B. cerinum ATCC 15112	CW-Tryp	0.25	0.68	0.23	0.23	0.23	0.24	0.47	0.40
		1.00	2.73	0.94	0.92	0.92	0.98	1.88	1.59
B. helvolum ATCC 11822	CW-Tryp	0.28	0.71	0.25	0.24	0.23	0.24	0.67	0.23
		1.00	2.59	0.92	0.88	0.84	0.88	2.48	0.84
Corynebacterium alkanum ATCC 21194	CW-Tryp	0.21	0.55	0.19	0.19	0.18	0.17	0.48	0.34
		1.00	2.69	0.93	0.93	0.90	0.82	2.35	1.67
Coryneform organism NCIB 8180	CW-Tryp	0.23	0.65	0.20	0.21	0.22	0.17	0.38	0.39
		1.00	2.83	0.87	0.89	0.95	0.71	1.63	1.67

[a] For abbreviations, see Table 1.
[b] The top number of each pair is amount expressed as micromoles per milligram of cell walls.
[c] The bottom number of each pair is amount relative to glutamic acid (Glu = 1).

of the bis-DNP-lysine demonstrated the D isomer in the case of glutamic acid and the L isomer in the case of lysine. Threonine and serine are present as L isomers because the test with D-amino acid oxidase was negative.

The configuration of alanine was enzymatically determined either in total hydrolysates of acid-extracted cell walls or after isolation of alanine by paper chromatography. CW-TCA were used to exclude the possibility that alanine-containing teichoic acids might falsify the results (1, 2).

As shown in Table 3, both isomers of alanine were found. D-Alanine occurs in a maximal amount of 1 mole per mole glutamic acid, and the ratio of L-Ala to D-Ala is always significantly above 1.0. The only exception is *Arthrobacter* sp. NCIB 9423 which shows 2 moles of D-Ala per mole of D-Glu and a ratio of L-Ala to D-Ala of about 1.

N-terminal and C-terminal amino acids. In the hydrolysates of dinitrophenylated cell walls, the DNP amino acids were determined qualitatively by thin-layer chromatography and quantitatively by comparison of the amino acid content of the hydrolysates of dinitrophenylated and non-dinitrophenylated cell walls. This method was used since the results

did not differ significantly from the photometric measurement (9).

As shown in Table 4, the only N-terminal amino acid in the murein of all strains tested is alanine, in an amount of 0.15 to 0.30 mole per mole muropeptide.

The C-terminal amino acids split off by hydrazinolysis were determined by the amino acid analyzer. To compensate for the incomplete hydrazinolysis on the one hand (9) and the destruction of released amino acids (4, 15, 17) on the other hand, the values were corrected by the factor 1.67.

The free amino acids arising from hydrazinolysis are alanine and a small amount of lysine. The total value agrees roughly with the amount of N-terminal alanine (Table 4).

Peptides from the partial hydrolysates of the different cell walls. To elucidate the primary structure of the mureins, the peptides of the partial acid hydrolysates (4 N HCl, 100 C, 30 or 60 min) were separated by two-dimensional paper chromatography and identified. Five different patterns of peptide maps (Fig. 1) indicating five different murein types were found. In the following these murein types are serially numbered from 1 to 5. These numbers are not correlated to chemotypes I to IV proposed by Ghuysen (7).

The detailed identification of the characteristic peptides of each murein type was carried out for the following strains: *A. aurescens* ATCC 13344 (murein type 1), *A. oxydans* ATCC 14358 (murein type 2), *A. citreus* ATCC 11524 (mu-

TABLE 3. *Configuration of alanine in the mureins of different strains of coryneform bacteria*

Strain	Moles/mole of Glu			L-Ala/D-Ala
	Total Ala	L-Ala	D-Ala	
Arthrobacter aurescens ATCC 13344	3.53	2.69	0.84	3.20
A. histidinolovorans ATCC 11442	3.70	3.03	0.67	4.56
Arthrobacter sp. ATCC 19141	3.50	2.81	0.69	4.05
A. ureafaciens ATCC 7562	3.52	2.91	0.61	4.81
Corynebacterium ilicis ATCC 14264	3.84	2.95	0.89	3.29
A. globiformis ATCC 8717	2.96	1.97	0.99	2.0
A. oxydans ATCC 14358	2.66	1.82	0.84	2.2
A. oxydans ATCC 14359	3.06	1.97	1.09	1.8
A. polychromogenes ATCC 15216	3.11	2.24	0.87	2.6
Arthrobacter sp. NCIB 9666	2.55	1.80	0.75	2.4
Coryneform organism NCIB 8180	2.97	2.23	0.74	3.0
A. citreus ATCC 11624	3.77	2.85	0.92	3.1
C. rathayi ATCC 13659	4.72	4.01	0.71	5.6
Arthrobacter sp. NCIB 9423	3.90	2.01	1.89	1.1

TABLE 4. *N- and C-terminal groups of the mureins of different strains of coryneform bacteria*

Strain	N-terminal Ala		C-terminal amino acids hydrazinolysis 80 C, 80 hr × 1.67[a]		
	CW-Tryp[b]	CW-TCA[b]	Ala	Lys	total
Arthrobacter aurescens ATCC 13344	0.26	0[c]	0.24	0.16	0.40
A. histidinolovorans ATCC 11442	0.11	0.39	0.28	0.09	0.37
Arthrobacter sp. ATCC 19141	0.12	0.11	0	0	0
A. ureafaciens ATCC 7562	0.15	0	0	0	0
Corynebacterium ilicis ATCC 14264	0.33	0.27	0	0	0
A. globiformis NCIB 8717	0.23	0	0	0	0
A. oxydans ATCC 14358	0.16	0.18	0.22	0.09	0.31
A. oxydans ATCC 14359	0.21	0.23	0	0	0
A. polychromogenes ATCC 15216	0.32	0.32	0	0	0
Coryneform organism NCIB 8180	0.24	0.22	0	0	0
A. citreus ATCC 11624	0.25	0.32	0.14	0.07	0.21
C. rathayi ATCC 13659	0.15	0.35	0.23	0.16	0.39
Arthrobacter sp. NCIB 9423	0.30	0	0.22	0.09	0.29

[a] The correction factor was determined by hydrazinolysis of authentic peptides.
[b] For abbreviations, see Table 1.
[c] 0, Not determined.

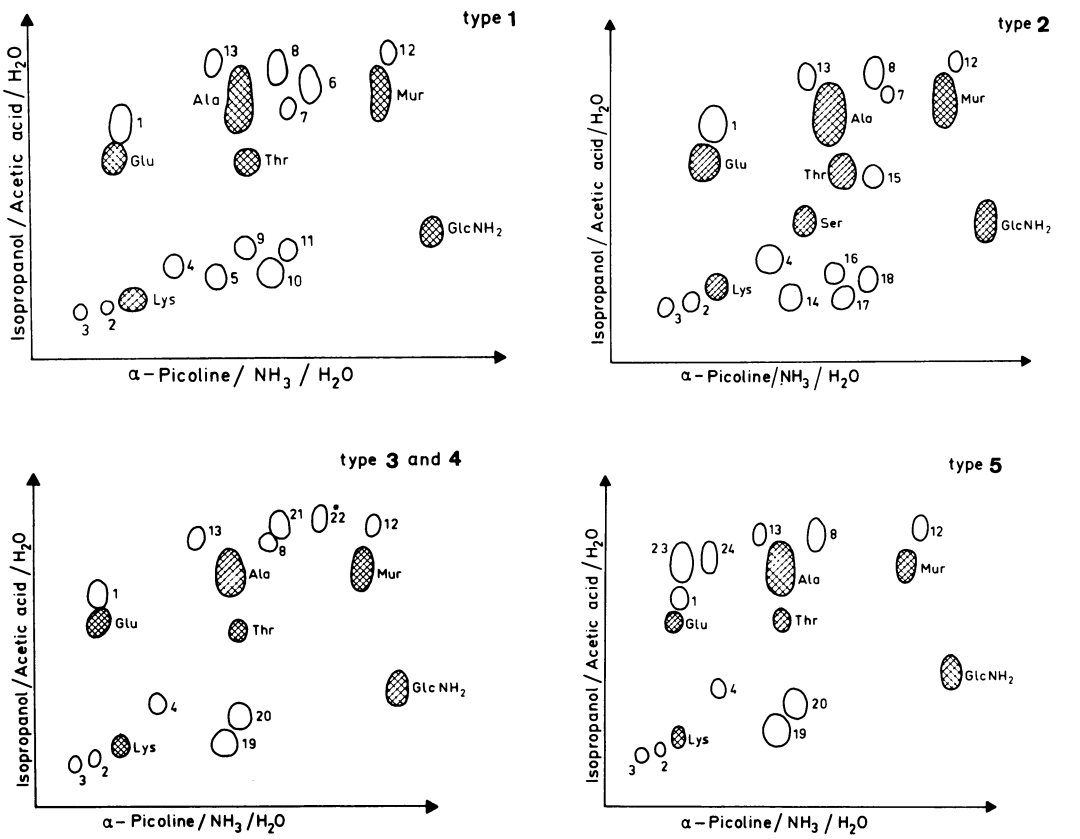

FIG. 1. *Appearance of the paper chromatograms of partial acid hydrolysates (4 N HCl, 100 C, 60 min) of cell walls of the five murein types. Peptides no. 12 and 13 appear in 30-min hydrolysates. *Peptide no. 22 occurs only in hydrolysates of cell walls of type 4; this distinguishes types 3 and 4.*

rein type 3), *C. rathayi* ATCC 13659 (murein type 4), and *Arthrobacter* sp. NCIB 9423 (murein type 5). The data for the peptides isolated from the partial hydrolysates are listed in Table 5.

DISCUSSION

The occurrence of the peptides Mur-L-Ala (no. 12), Mur-L-Ala-D-Glu (no. 13), L-Ala-D-Glu (no. 1), γ-D-Glu-L-Lys (no. 2), L-Lys-D-Ala (no. 4), and γ-D-Glu-L-Lys-D-Ala (no. 3) shows that the peptide subunit L-Ala-D-Glu-L-Lys-D-Ala bound to muramic acid is common in all murein types described in this paper. In the case of the murein type 5, the α-carboxyl group of D-glutamic acid of the peptide subunit is substituted by D-alanine as indicated by the appearance of the peptides α-D-Glu-D-Ala (no. 23) and L-Ala—$\overset{\alpha}{}$—D-Glu-D-Ala (no. 24). The various patterns of the remaining peptides (Fig. 1 and Table 5) reflect the different amino acid sequences of the interpeptide bridges of the five murein types.

For purposes of description, the murein type is characterized by a simplified characteristic fragment of the structure as proposed by Schleifer and Kandler (Bacteriol. Rev., *in press*). It consists of the L-lysine of the peptide subunit and the attached interpeptide bridge. Insofar as the interpeptide bridge is bound through its C-terminus to the ε-amino group of lysine, this representation of the amino acid sequence of the interpeptide bridge is the reverse of the accepted convention in which peptides are written beginning with the N-terminus.

Murein type 1: L-Lys-L-Ala-L-Thr-L-Ala. The interlinkage between the ε-amino group of L-lysine and the C-terminal D-alanine of an adjacent peptide subunit is mediated by the tripeptide L-Ala-L-Thr-L-Ala. This was deduced from the appearance of the peptides ε-L-Ala-L-Lys (no. 5), L-Thr-L-Ala (no. 6), L-Ala-L-Thr (no. 7), D-Ala-L-Ala (no. 8), ε-L-Ala-L-Lys-D-Ala (no. 9), ε-(L-Thr-L-Ala)-L-Lys (no. 10), and ε-(L-Thr-L-Ala)-L-Lys-D-Ala (no. 11).

TABLE 5. *Characterization of the peptides of the partial acid hydrolysates of cell walls of the five murein types separated by paper chromatography*

No. of peptide	R_{Ala}		Molar ratio						Product of the hydrolysis of the DNP-peptides	Configuration of Ala	Amino acid sequence
	Solvent I	Solvent II	Glu	Lys	Ala	Thr	Ser	Mur			
1	0.96	0.34	1		1				DNP-Ala	L	L-Ala-D-Glu
2	0.27	0.28	1	1					ε-DNP-Lys, DNP-Glu		γ-D-Glu-L-Lys
3	0.26	0.18	1	1	1				DNP-Glu, ε-DNP-Lys	D	γ-D-Glu-L-Lys-D-Ala
4	0.44	0.64		1	1				bis-DNP-Lys	D	L-Lys-D-Ala
5	0.39	0.86		1	1				DNP-Ala, α-DNP-Lys	L	ε-L-Ala-L-Lys
6[a]	1.10	1.32			1	1			DNP-Thr		L-Thr-L-Ala
7	1.00	1.25			1				DNP-Ala	0[b]	L-Ala-L-Thr
8	1.16	1.20			1				0[b]	DL	D-Ala-L-Ala
9	0.53	1.00		1	2				DNP-Ala, α-DNP-Lys	DL	ε-L-Ala-L-Lys-D-Ala
10	0.40	1.11		1	1	1			DNP-Thr, α-DNP-Lys	L	ε-(L-Thr-L-Ala)-L-Lys
11	0.50	1.18		1	2	1			DNP-Thr, α-DNP-Lys	DL	ε-(L-Thr-L-Ala)-L-Lys-D-Ala
12	1.21	1.67			1			1	0	L	Mur-L-Ala
13	1.19	0.88	1		1			1	0	L	Mur-L-Ala-D-Glu
14	0.30	0.82					1		DNP-Ser-α-DNP-Lys		ε-L-Ser-L-Lys
15[a]	0.77	1.18				1	1		DNP-Thr		L-Thr-L-Ser
16	0.40	0.96		1	1		1		DNP-Ser-α-DNP-Lys	D	ε-L-Ser-L-Lys-D-Ala
17	0.29	1.05		1		1	1		DNP-Thr-α-DNP-Lys		ε-(L-Thr-L-Ser)-L-Lys
18	0.37	1.13		1	1	1	1		DNP-Thr-α-DNP-Lys	D	ε-(L-Thr-L-Ser)-L-Lys-D-Ala
19	0.28	0.95		1		1			DNP-Thr-α-DNP-Lys		ε-L-Thr-L-Lys
20	0.39	1.04		1	1	1			DNP-Thr-α-DNP-Lys	D	ε-L-Thr-L-Lys-D-Ala
21	1.22	1.25			1				0	L	L-Ala-L-Ala
22	1.24	1.42			1				0	L	L-Ala-L-Ala-L-Ala
23	1.09	0.49	1		1				DNP-Glu	D	α-D-Glu-D-Ala
24	1.14	0.63	1		2				DNP-Ala	DL	L-Ala-α-D-Glu-D-Ala

[a] Peptides no. 6 and 15 appear yellow after spraying with ninhydrin.
[b] 0, Not determined.

Murein type 2: L-Lys-L-Ser-L-Thr-L-Ala. The mode of cross-linkage is very similar to type 1. The appearance of the peptides ε-L-Ser-L-Lys (no. 14) and ε-L-Ser-L-Lys-D-Ala (no. 16) indicates that in this case L-serine substitutes the ε-amino group of L-lysine instead of L-alanine. The remaining portion of the interpeptide bridge is the same as in murein type 1. This is evident from the occurrence of the peptides L-Thr-L-Ser (no. 16), L-Ala-L-Thr (no. 7), D-Ala-L-Ala (no. 8), ε-(L-Thr-L-Ser)-L-Lys (no. 17), and ε-(L-Thr-L-Ser)-L-Lys-D-Ala (no. 18). The interpeptide bridge consists, therefore, of the peptide L-Ala-L-Thr-L-Ser.

Murein types 3, 4, and 5: L-Lys-L-Thr-L-Ala_2, L-Lys-L-Thr-L-Ala_3, L-Lys-L-Thr-L-Ala. In the murein types 3, 4, and 5 L-threonine is bound to the ε-amino group of L-lysine. This is demonstrated by the existence of the relatively stable peptide ε-L-Thr-L-Lys (no. 19) as well as by the tripeptide ε-L-Thr-L-Lys-D-Ala (no. 20). The further amino acid sequence of the interpeptide bridges and the linkage to the C-terminal D-alanine of an adjacent peptide subunit is shown by the peptides L-Ala-L-Ala (no. 21) and D-Ala-L-Ala (no. 8) in the case of type 3; by L-Ala-L-Ala (no. 21), L-Ala-L-Ala-L-Ala (no. 22), and D-Ala-L-Ala (no. 8) in the case of type 4; and finally by L-Ala-D-Ala (no. 8) only in the case of type 5.

In agreement with the amount of alanine in the total cell wall, the interpeptide bridge of type 3 consists of the tripeptide L-Ala-L-Ala-L-Thr, that of type 4 of the tetrapeptide L-Ala-L-Ala-L-Ala-L-Thr, and that of type 5 consists of the dipeptide L-Ala-L-Thr.

The dipeptide L-Ala-L-Thr, which should be found, was actually not present in the partial hydolysates. It could only be detected in traces in partial hydolysates of cell walls of types 1 and 2. The very poor yield of this peptide is not surprising since the peptide bound to a threonyl or a seryl residue (X-Thr or X-Ser) is extremely sensitive to acid hydrolysis (6).

As mentioned, all of the mureins contain about 1 mole of ammonia per mole of muropeptide which is split off during short hydrolysis (4 N HCl, 100 C, 1 hr). The only possible position of this amide group is the α-carboxyl group of D-glutamic acid in the cases of the murein types 1, 2, 3, and 4. Such an amidation of the α-carboxyl group of D-glutamic acid is known for other mureins (7). In the case of type 5, the carboxyl group of D-alanine substituting the α-carboxyl group of D-glutamic acid is amidated. The occurrence of D-alanine amide agrees

with the results of the hydrazinolysis (Table 4). If D-alanine were not amidated, a large amount of free alanine should arise during the hydrazinolysis, whereas only small amounts were found, as in the other strains. The primary structures of the five threonine-containing murein types can be written as shown in Fig. 2 and 3. For each murein type, only one representative strain was studied in detail (*see above*). From all the other strains only the molar ratios of the amino acids and amino sugars were determined, and the respective two-dimensional paper chromatograms of the acid partial hydrolysates were compared with those strains studied in detail. When peptide maps were exactly identical, we assumed that the amino acid sequence was also identical. The distribution of the murein types among the 21 strains investigated is given in Table 6.

The schemes of the primary structure of the murein types (Fig. 2 and 3) are not in full agreement with the data of the Tables 3 and 4. The genuine mureins differ from them as follows.

(i) The occurrence of about 0.15 to 0.30 mole of N-terminal alanine per mole of glutamic acid shows that about 15 to 30% of interpeptide bridges are not cross-linked. Since only DNP-alanine, and neither DNP-threonine nor DNP-serine nor ε-DNP-lysine, is found in the hydrolysates of DNP-cell walls, it is likely that no incomplete interpeptide chains are present in the mureins.

(ii) In agreement with the incomplete cross-linking, some D-alanine of the peptide subunits is C-terminal (Table 4). In addition to this, there is about 10% C-terminal lysine. The loss of the C-terminal D-alanine from the uncross-linked peptide subunits is very common in

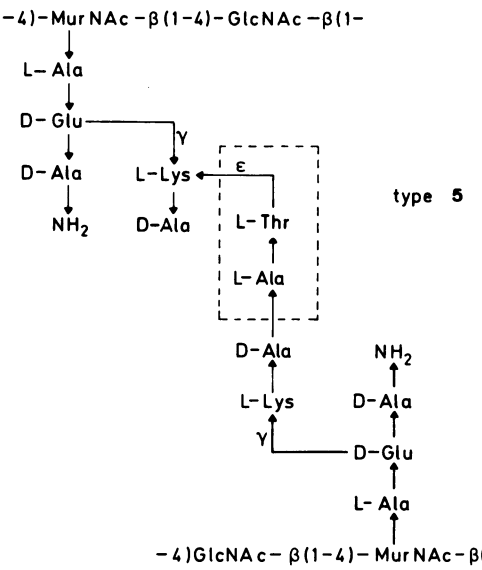

FIG. 3. *Fragment of the primary structure of the murein of the murein type 5. The interpeptide bridge is marked by a dashed frame.*

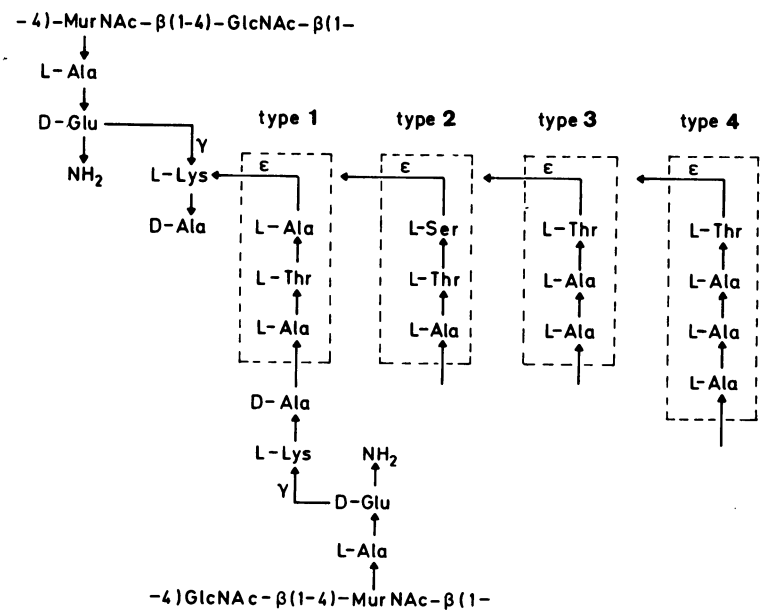

FIG. 2. *Fragments of the primary structure of the mureins of type 1, 2, 3 and 4. The interpeptide bridges are marked by dashed frames.*

TABLE 6. *Distribution of the murein types 1, 2, 3, 4, and 5 among coryneform bacteria*[a]

Murein type	Organisms
Type 1 L-Lys-L-Ala-L-Thr-L-Ala	*A. aurescens* ATCC 13344, *A. histidinolovorans* ATCC 11442, *A. ureafaciens* ATCC 7562, *Arthrobacter* sp. ATCC 19141, *B. helvolum* ATCC 19239, *C. ilicis* ATCC 14264,
Type 2 L-Lys-L-Ser-L-Thr-L-Ala	*A. globiformis* NCIB 8717, *A. oxydans* ATCC 14358, *A. oxydans* ATCC 14359, *A. polychromogenes* ATCC 15216, *Arthrobacter* sp. NCIB 9666, *B. album* ATCC 15111, *B. ammoniagenes* ATCC 6871, *B. cerinum* ATCC 15112, *B. helvolum* ATCC 11822, *C. alkanum* ATCC 21194, coryneform organism NCIB 8180,
Type 3 L-Lys-L-Thr-$L\text{-Ala}_2$	*A. citreus* ATCC 11624
Type 4 L-Lys-L-Thr-$L\text{-Ala}_3$	*C. rathayi* ATCC 13659, *Corynebacterium* sp. ATCC 21188
Type 5 L-Lys-L-Thr-L-Ala	*Arthrobacter* sp. NCIB 9423

[a] The fragments of the primary structure are depicted in Fig. 2.

other mureins also (7). It is probably caused by the action of a carboxypeptidase leading to a peptide subunit with C-terminal L-lysine. In good agreement with this observation, the ratio of D-Ala to L-Ala (Table 3) is always lower than expected, since some D-alanine is missing. If these deviations in the primary structure of the mureins are taken into account, the data in Tables 3 and 4 are in agreement with the schemes drawn in Fig. 2 and 3.

With the exception of the Lys-Thr-Ala_3 murein type (type 4) which was found in *Micrococcus roseus* earlier (14), all of the other murein types were not known before. In the case of murein type 5 the interpeptide bridge is identical with that of the mureins of *Streptococcus cremoris* (20) and *Streptotoccus bovis* (11), but in the latter mureins the α-carboxyl group of glutamic acid is substituted by an amide, whereas in type 5 it is substituted by a D-alanine amide. A similar substitution by an amino acid amide has been found up to now only in the case of *A. atrocyaneus;* there glycineamide is present instead of D-alanine amide (9).

These threonine-containing mureins are a further example that the determination of the qualitative and even the quantitative amino acid composition of the cell wall is not sufficient for an unequivocal characterization of the type of murein (10). The quantitative amino acid compositions of the mureins of types 1, 3, and 5 (Glu:Lys:Thr:Ala = approximately 1:1:1:4) are identical. But the partial hydrolysates of these cell walls clearly demonstrate different peptide patterns (Fig. 1) and indicate that three distinct amino acid sequences are present.

We are well aware that the distribution of these different murein types among coryneform bacteria is certainly of taxonomic value. Before a new proposal for the subdivision of the coryneforms can be made, the correlation between the murein structure and other characters has to be elaborated.

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