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A New Type of Peptide Subunit in the Murein of *Arthrobacter* Strain J39*

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ABSTRACT: The murein (peptidoglycan) of *Arthrobacter* strain J39 was found to contain D-alanine, L-lysine, glycine, and D- and L-glutamic acid in the molar ratio of 1:1:3:2. Approximately half of the D-glutamic acid was hydroxylated. The UDP-activated precursors of the murein were isolated. The molar ratios Mur:Gly:Glu = 1:1:2 and Mur:Gly:Glu:Ala = 1:1:2:2 were found for the tripeptide and the pentapeptide, respectively. Partial acid hydrolysis of the purified precursors and analysis of the obtained peptides proved the sequence *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamic acid for the UDP-*N*-acetylmuramyl tripeptide, and the sequence *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamyl-D-alanyl-D-alanine for the UDP-*N*-acetylmuramyl pentapeptide. Analysis of the peptides isolated from partial acid hydrolysates of cell walls confirmed the amino acid sequence of the peptide subunit as found in the murein precursors. The cross-linkage of the peptide subunits was shown to be performed by the peptide glycylglycyl-L-lysine. The *N*-terminal glycine is attached to the α-carboxyl group of the D-glutamic acid, while the ε-amino group of the lysine is bound to the D-alanine of the adjacent peptide subunit.

Most mureins (peptidoglycans) contain only 1 mole of glutamic acid/mole of peptide subunit. When additional glutamic acid is found, it is localized in the interpeptide bridge (Niebler *et al.*, 1969; Kandler *et al.*, 1970; Schleifer and Kandler, 1970; D. Bogdanovsky *et al.*, 1971, in preparation). The cell wall of an *Arthrobacter* isolated by Keddie *et al.* (1966) was found to contain 2 moles of glutamic acid/mole of peptide subunit. The present paper describes the amino acid sequence of this murein. Unlike the other mureins which are rich in glutamic acid, in this case the second mole of glutamic acid is not a constituent of the interpeptide bridge, but of the peptide subunit.

Material and Methods

The *Arthrobacter* strain J39 was kindly supplied by Dr. Keddie, Department of Microbiology, The University, Reading, England. The bacteria were grown aerobically in yeast extract glucose broth (Schleifer *et al.*, 1967a) with 0.5% NaCl at 28°. Cell walls were prepared by the usual technique (Schleifer and Kandler, 1967).

Total hydrolysis of cell walls, peptides, and murein precursors was carried out in 4 N HCl at 100° for 16 hr or in 5 N HCl at 100° for 5 hr. Partial acid hydrolysis was performed as described previously (Schleifer and Kandler, 1967).

The following solvent systems were used for separation of amino acids, amino sugars, and peptides by paper chromatography: (I) isopropyl alcohol–acetic acid–water (75:10:15, v/v) and (II) α-picoline–25% NH_4OH–water (70:2:28, v/v).

Peptides containing hydroxyglutamic acid and glutamic acid were separated by high-voltage electrophoresis under the following conditions: 58 V/cm, 2–4 hr, formic acid–acetic acid–water (5:15:80, v/v), pH 1.9, Whatman paper No. 3MM (Schleifer *et al.*, 1967b).

Quantitative determination of amino acids was performed with an amino acid analyzer.

Dinitrophenylation of cell walls and peptides was carried out according to Primosigh *et al.* (1961). The dinitrophenylated amino acids were identified by paper chromatography

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TABLE I: Absolute Amounts and Molar Ratios of Amino Acids and Amino Sugars of Three Different Cell Wall Preparations (A, B, and C) of Strain J39.

	μmole/mg of Cell Wall			Molar Ratio Glu + Hyg = 2		
	A	B	C	A	B	C
Mur	0.149	0.112	0.168	0.91	0.79	0.84
Glu	0.327	0.227	0.312	1.53	1.60	1.55
Hyg	0.100	0.058	0.090	0.47	0.40	0.45
Gly	0.655	0.433	0.594	3.06	3.04	2.96
Ala	0.191	0.133	0.177	0.89	0.94	0.88
Lys	0.240	0.155	0.216	1.12	1.09	1.07
$GlcNH_2$	0.206	0.136	0.237	0.96	0.96	1.18
NH_3	0.127	0.097	0.084	0.60	0.68	0.42
Ser	0.027	0.021	0.027	0.13	0.15	0.13

using the following systems: (III) 1.5 M phosphate buffer (pH 6.0) (Levy, 1954) and (IV) *n*-propyl alcohol–0.2% ammonia (8:2, v/v) (Braunitzer, 1955).

Determination of C-terminal amino acids was performed by hydrolysis with anhydrous hydrazine as described by Braunitzer (1955).

Optical rotatory dispersion of the di-DNP derivate was used to determine the configuration of lysine (Kandler *et al.*, 1968).

The quantitative determination of the L and D isomers of alanine and of L-glutamic acid was carried out enzymatically (Niebler *et al.*, 1969). Photolysis of the dinitrophenylated peptides was used to demonstrate whether α- or γ-bound glutamic acid was present (Perkins, 1967; Niebler *et al.*, 1969).

UDP-activated murein precursors were isolated as follows. Overnight cultures were diluted into 10 volumes of fresh medium and incubated under aerobic conditions until they had reached an optical density of 1.0 at 578 mμ. For accumulation of UDP-*N*-acetylmuramyl tripeptide, 100 μg/ml of D-cycloserine (Deutsche Hoffmann-LaRoche AG, Grenzach) was added and the culture was further incubated for 1.5 hr (Roze and Strominger, 1966). The cells were harvested by centrifugation, extracted with 25% (w/v) trichloroacetic acid at 4° for 30 min, and centrifuged (Chatterjee and Perkins, 1966). The supernatant after ether extraction of trichloroacetic acid was neutralized, concentrated under vacuum at room temperature, and applied to a column (80 × 2.5 cm) of Sephadex G-25 following the procedure of Rosenthal and Sharon (1964). Elution was carried out with 0.01 N acetic acid. Fractions absorbing at 260 mμ and containing bound *N*-acetylamino sugar (Reissig *et al.*, 1955) were pooled, concentrated, and chromatographed on Whatman No. 3MM paper in isobutyric acid–0.5 M ammonia (5:3, v/v) as described by Chatterjee and Perkins (1966).

Results

Amino Acid Composition of the Murein. Cell walls of three different preparations were examined (Table I).

The *threo*-3-hydroxyglutamic acid was identified by determining the R_F values on paper chromatograms and the elution volume on the amino acid analyzer (Schleifer *et al.*, 1967b).

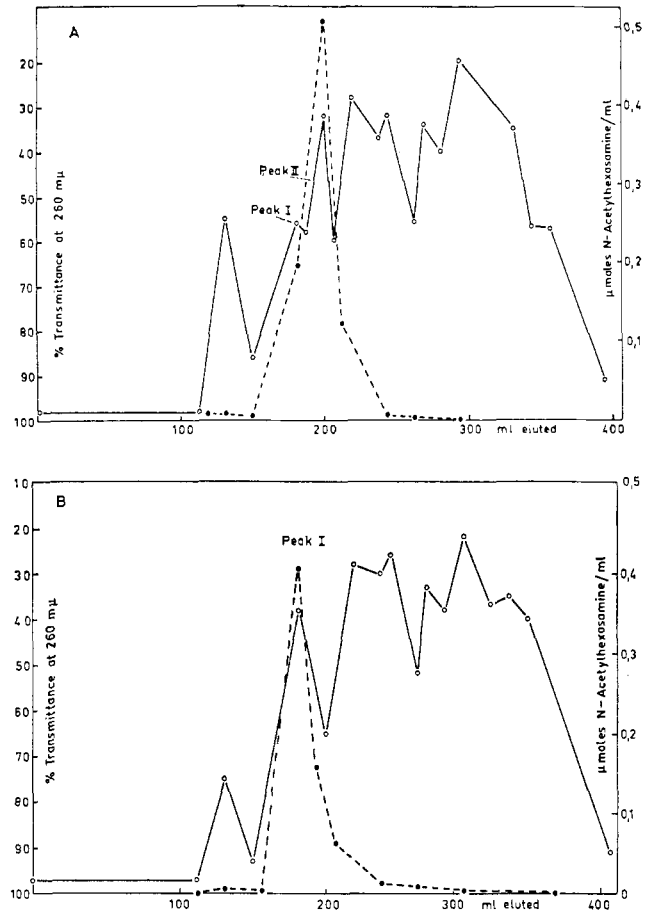

FIGURE 1: Separation of trichloroacetic acid extract on Sephadex G-25. (A) + D-Cycloserine. (B) Without antibiotic. Solid line: per cent transmittance at 260 mμ. Dashed line: *N*-acetylhexosamine.

The release of small amounts of ammonia (less than 1 mole/mole of peptide subunit) after total acid hydrolysis of the cell walls is caused by the destruction of amino sugars and not by the release of amide ammonia. This was demonstrated by measuring the release of ammonia by short-time hydrolysis in 4 N HCl at 100°. Only 0.3 mole of ammonia/mole of peptide subunit was found after 4 hr and only traces were found after 1 hr.

Lysine was present in the L configuration and alanine in the D configuration. The determination of the isomers of glutamic acid yielded 1 mole of L-glutamic acid and 10.43 mole of D-glutamic acid per mole of peptide subunit.

Determination of N- and C-Terminal Amino Acids. The total hydrolysate of the dinitrophenylated cell walls was separated by thin-layer chromatography on cellulose in solvent system III. The only DNP-amino acid found was ε-DNP-lysine. The quantitative determination of the amino acids of dinitrophenylated cell walls showed a decrease in lysine of about 15% compared to undinitrophenylated cell walls. Hydrazinolysis showed that only lysine is C terminal in considerable amounts.

Isolation of the Murein Precursors. Lysine was found to be C terminal as well as N terminal. This suggests that lysine is not a constituent of the peptide subunit but participates in the cross-linkage as found in some other coryneform organisms (Schleifer, 1970a; Fiedler *et al.*, 1970). Consequently the normal position of lysine within the peptide subunit had to be

TABLE II: Amino Acid Composition of the Ultraviolet-Absorbing Material, Isolated from Peaks I and II of the Eluate from the Sephadex Column.

	μmole/Sample				Molar Ratio Glu = 2		
	Mur	Glu	Gly	Ala	Mur	Gly	Ala
Peak I	0.076	0.138	0.075	0.133	1.1	1.09	1.93
Peak II	0.314	0.579	0.276		1.09	0.95	

occupied by another amino acid, probably L-glutamic acid. To verify this assumption, we isolated the UDP-activated murein precursors.

The separation of the trichloroacetic acid extract of bacteria treated with D-cycloserine by column chromatography on Sephadex resulted in two uv-absorbing peaks (I and II), which contained nearly all *N*-acetylamino sugar nucleotides (Figure 1). The extract of the culture grown without antibiotic yielded only peak I.

After paper chromatography the bulk of uv-absorbing material of peak I showed a R_{ATP} of 0.6, the bulk of uv-absorbing material of peak II showed a R_{ATP} of 0.4. There was no color reaction with ninhydrin.

The uv-absorbing bands were eluted. An aliquot was hydrolyzed (5 N HCl, 100°, 5 hr) and analyzed for amino acids. As seen in Table II the molar ratios indicate that peak I contained mainly nucleotide pentapeptide, peak II nucleotide tripeptide. Hydroxyglutamic acid was not found in the precursors. The glutamic acid seems to become hydroxylated later in the biosynthesis of the murein (Schleifer *et al.*, 1967a). Determination of the configuration of glutamic acid of the pentapeptide yielded 1.08 moles of the L isomer and 0.97 mole of the D isomer. Alanine was found only in D configuration.

The partial acid hydrolysates of both precursors were

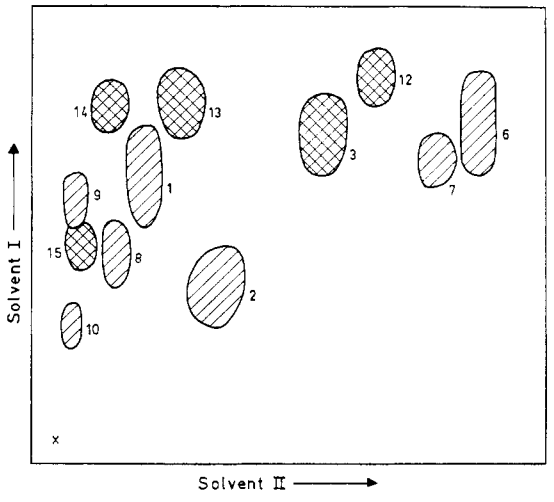

FIGURE 2: Scheme of a paper chromatogram of a 5-min partial acid hydrolysate of the UDP-*N*-acetylmuramyl pentapeptide and the *N*-acetylmuramyl tripeptide isolated from strain J39. 1 = glutamic acid; 2 = glycine; 3 = alanine; 6 = muramic acid; 7 = MurGly; 8 = Gly-D-Glu; 9 = γ-D-Glu-L-Glu; 10 = Gly-γ-D-Glu-L-Glu; 12 = D-Ala-D-Ala; 13 = L-Glu-D-Ala-D-Ala; 14 = L-Glu-D-Ala; 15 = Gly-γ-D-Glu-L-Glu-D-Ala-D-Ala; crosshatch: amino acids and peptides only found in the hydrolysates of the UDP-*N*-acetylmuramyl pentapeptide.

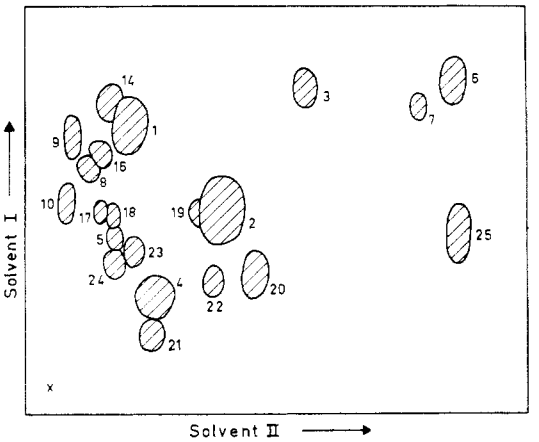

FIGURE 3: Scheme of a paper chromatogram of a partial acid hydrolysate of the cell wall of strain J39. 1 = glutamic acid; 2 = glycine; 3 = alanine; 4 = lysine; 5 = *threo*-3-hydroxyglutamic acid; 6 = muramic acid; 7 = MurGly; 8 = Gly-D-Glu; 9 = γ-D-Glu-L-Glu; 10 = Gly-γ-D-Glu-L-Glu; 14 = L-Glu-D-Ala; 16 = D-Glu-Gly; 17 = Gly-D-Glu-Gly and GlyHygGly; 18 = GlyHyg; 19 = GlyGly; 20 = N^6-D-Ala-L-Lys; 21 = N^2-Gly-L-Lys; 22 = N^2-Gly-N^6-D-Ala-L-Lys; 23 = N^6-(L-Glu-D-Ala)-L-Lys; 24 = N^2-Gly-N^6-(L-Glu-D-Ala)-L-Lys; 25 = *N*-acetylglucosamine.

separated by two-dimensional paper chromatography in solvent systems I and II (Figure 2). The results of the amino acid sequence determination of the peptides are shown in Table III. The peptides muramylglycine (7), glycyl-D-glutamic acid (8), γ-D-glutamyl-L-glutamic acid (9), and glycyl-γ-D-glutamyl-L-glutamic acid (10) prove the following structure of the *N*-acetylmuramyl tripeptide: *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamic acid. In the partial acid hydrolysates of the pentapeptide precursor D-alanine as well as the peptides D-alanyl-D-alanine (12), L-glutamyl-D-alanyl-D-alanine (13), L-glutamyl-D-alanine (14), and glycyl-γ-D-glutamyl-L-glutamyl-D-alanyl-D-alanine (15) were found in addition. Thus the *N*-acetylmuramyl pentapeptide shows the structure *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamyl-D-alanyl-D-alanine.

The Amino Acid Sequence of the Murein. Partial acid hydrolysates of cell walls were separated by paper chromatography (Figure 3). The peptides were isolated and identified. The results are shown in Table IV.

Analysis of the precursors had proved the structure of the peptide subunit to be *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamyl-D-alanine. This was confirmed by the peptides 8, 9, 10, and 14 isolated from partial acid hydrolysates of cell walls. Since only peptides containing hydroxyglutamic acid and glycine, rather than hydroxyglutamic acid and alanine, were detected, only D-glutamic acid is hydroxylated. The ratio of D-glutamic acid to L-glutamic acid was about 0.5:1 instead of 1:1 as determined in the precursor. This indicates that about half of the D-glutamic acid is hydroxylated.

The cross-linkage occurs between the α-carboxyl group of the D-glutamic acid and the D-alanine of an adjacent peptide subunit. It is performed by the peptide glycylglycyl-L-lysine. The occurrence of a diglycine in the partial acid hydrolysates indicates that the α-carboxyl group of the glutamic acid is substituted by 2 moles of glycine. Since only 1 mole of glycine was detected in the precursor, but 3 moles of glycine are present per mole of lysine, one must assume that 2 moles of glycine are present in the interpeptide bridge.

Glycine is bound to the α-carboxyl group of the D-glutamic

TABLE III: Amino Acid Composition and Structure of the Peptides Isolated from Partial Acid Hydrolysates of the Murein Precursors of Strain J39.

Peptide No.	Amino Acid Composition (μmole/Sample)	Molar Ratio	Terminal Groups Configuration Photolysis of the DNP Derivative	Peptide Structure
7	Mur, 0.050	1	Gly C terminal	MurGly
	Gly, 0.049	1		
8	Gly, 0.034	0.9	Gly N terminal	Gly-D-Glu
	Glu, 0.038	1	D-Glutamic acid	
9	Glu only		Photolysis positive	γ-D-Glu-L-Glu
10	Gly, 0.018	1	Gly N terminal	Gly-γ-D-Glu-L-Glu
	Glu, 0.039	2		
12	Ala only			D-Ala-D-Ala[a]
13	Glu, 0.085	1	Photolysis negative	L-Glu-D-Ala-D-Ala
	Ala, 0.172			
14	Glu, 0.135	1	L-Glu N terminal	L-Glu-D-Ala
	Ala, 0.136	1		
15	Gly, 0.031	1	Gly N terminal	Gly-γ-D-Glu-L-Glu-D-Ala-D-Ala
	Glu, 0.066	2		
	Ala, 0.063	2		

[a] Schleifer *et al.* (1969).

TABLE IV: Amino Acid Composition and Structure of Peptides Isolated from Partial Acid Hydrolysates of Cell Walls of Strain J39.

Peptide No.	Amino Acids (μmole/Sample)	Molar Ratio	Terminal Groups Configuration	Partial Acid Hydrolysate of the Peptide	Peptide Structure
8	Glu, 0.036	1	Gly N terminal		Gly-D-Glu
	Gly, 0.035	1	D-Glu		
9	Glu only				γ-D-Glu-L-Glu
10	Glu, 0.112	1.8	Gly N terminal	Glu,Gly,Hgy, GlyGlu, GlyHyg	Gly-γ-Hyg-L-Glu and Gly-γ-D-Glu-L-Glu
	Hyg, 0.034	0.4			
	Gly, 0.063	1			
14	Glu, 0.046	1	Glu N terminal		L-Glu-D-Ala
	Ala, 0.045	1	L-Glu, D-Ala		
16	Glu, 0.059	1	Glu N terminal		D-GluGly[a]
	Gly, 0.057	1	D-Glu		
17	Glu, 0.018	1	Gly N terminal		Gly-D-GluGly
	Gly, 0.040	2.2			
18	Hyg, 0.046	0.7	Gly N terminal		GlyHyg
	Gly, 0.067	1			
19	Gly only				GlyGly[b]
20	Ala, 0.051	1	Ala N terminal		N^6-D-Ala-L-Lys[c]
	Lys, 0.048	0.9			
21	Gly, 0.067	1	Gly N terminal		N^2-Gly-L-Lys[c]
	Lys, 0.089	1.3			
22	Gly, 0.033	1.3	Gly N terminal		N^2-Gly-N^6-D-Ala-L-Lys
	Ala, 0.026	1			
	Lys, 0.024	1			
23	Glu, 0.033	1		Glu,Lys,Ala,GluAla,N^6-Ala-Lys	N^6-(L-Glu-D-Ala)-L-Lys
	Ala, 0.035	1			
	Lys, 0.033	1			
24	Glu, 0.011	0.7	Gly N terminal	Gly,Glu, L-Glu-D-Ala,GlyLys, N^6-Ala-Lys	N^2-Gly-N^6-(L-Glu-D-Ala)-L-Lys
	Gly, 0.015	1			
	Ala, 0.015	1			
	Lys, 0.014	0.9			

[a] Schleifer (1970b). [b] Schleifer *et al.* (1969). [c] Schleifer *et al.* (1968).

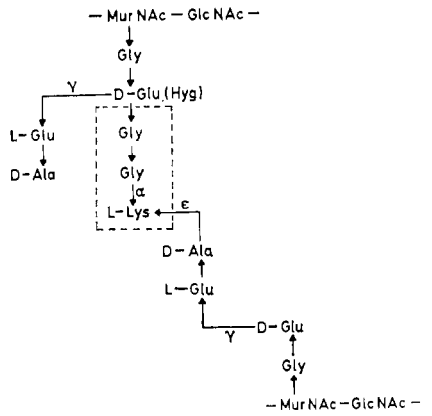

FIGURE 4: Fragment of the primary structure of the murein of strain J39. (Interpeptide bridge marked by a dashed frame.)

acid, as shown by the occurrence of the peptides D-glutamylglycine (16) and glycyl-D-glutamylglycine (17). The α-amino group of lysine is bound to glycine as demonstrated by the occurrence of the peptide N^2-glycyl-L-lysine (21). The ϵ-amino group of lysine is attached to the carboxyl group of a D-alanine. This could be shown by the peptides N^6-D-alanyl-L-lysine (20) and N^2-glycyl-N^6-D-alanyl-L-lysine (22).

A fragment of the primary structure of the murein is depicted in Figure 4. In the different preparations of cell walls 0.1 mole of serine was found per mole of peptide subunit. Small amounts of peptides containing serine, hydroxyglutamic acid, and glycine were also found in the partial acid hydrolysates of the cell walls. This indicates that serine replaces some glycine in the murein.

Discussion

It is well known that the most variable part of the murein is the interpeptide bridge which contains several mono- and dicarboxylic amino acids in many combinations (Schleifer, 1970a; Fiedler *et al.*, 1970; Kandler *et al.*, 1970). It even contains diamino acids, when the cross-linkage occurs between the α-carboxyl group of the glutamic acid and the C-terminal D-alanine of an adjacent peptide subunit. On the other hand, the composition of the peptide subunit is much more constant.

The usual L-alanine in the first position of the peptide subunit is replaced by glycine in some coryneform organisms (Fiedler *et al.*, 1970) or by serine in *Butyribacterium rettgeri* (Guinand *et al.*, 1969). The only variation known in position 2 is the hydroxylation of the D-glutamic acid (Schleifer *et al.*, 1968). The most frequent variation occurs in position 3, but usually only various diamino acids, namely, *m*-DAP,[1] LL-DAP, lysine, ornithine, or DAB are involved. A few coryneform bacteria containing L-homoserine instead of a diamino acid (Perkins, 1967; Fiedler *et al.*, 1970; Perkins, 1971) are rare exceptions. J39, described in this paper, is the first case where a dicarboxylic amino acid occupies position 3. As in all other cases this glutamic acid also is of the L configuration.

Thus it is again confirmed that only L-amino acids or the L center of an amino acid occurs at position 3. Consequently, the sequence of alternating D- and L-amino acids in the peptide subunit is maintained in this otherwise unique murein.

[1] Abbreviations used are: DAP, 2,6-diaminopimelic acid; DAB, 2,4-diaminobutyric acid; Hyg, *threo*-3-hydroxyglutamic acid.

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