Autolysis of Cell Walls of *Bacillus Stearothermophilus* B65 and the Chemical Structure of the Peptidoglycan

By W. D. GRANT AND A. J. WICKEN

School of Microbiology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia

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1. The cell walls of *Bacillus stearothermophilus* B65 contain glucosamine, muramic acid, alanine, $\alpha\epsilon$ -diaminopimelic acid (Dap), glutamic acid, aspartic acid, glycine, and serine in the molecular proportions 0.60:0.64:2.30:0.85:1.00:0.11:0.13:0.31. 2. Both D- and L-alanine are present, but glutamic acid and diaminopimelic acid are present only as the D- and *meso*-isomers respectively. 3. The peptide fragments Ala-Dap, Dap-Ala, and Dap-Ala-Dap have been isolated from a partial acid hydrolysate of the cell walls. 4. The major products of autolysis of the cell wall were D-alanine, a peptide mixture, peptidoglycan material and a peptidoglycan-teichoic acid complex. 5. Separation of the peptide mixture into ten major peptides was achieved by DEAE-Sephadex and paper chromatography, and paper electrophoresis. 6. The structures of these peptides have been determined and they fall into four groups, the individual members of each group differing only in number or position of carboxamide substituents. 7. The structures are I, a tripeptide L-Ala-D-Glu-*meso*-Dap; II, a pentapeptide made up by the tripeptide (I) linked through the ϵ -amino group of its diaminopimelic acid residue to the carboxy-terminal of the dipeptide *meso*-Dap-D-Ala; III, a heptapeptide made up by a similar linkage between the tripeptide (I) and the tetrapeptide L-Ala-D-Glu-*meso*-Dap-D-Ala; IV, a possible undecapeptide made up by a further tetrapeptide similarly linked to the heptapeptide (III) structure. 8. The structure of the peptidoglycan, and the actions of the autolytic enzymes are discussed in terms of these peptide structures.

Two major difficulties in structural studies on bacterial cell-wall peptidoglycan have been the separation of the polymer from other components of the wall structure, such as polysaccharide and teichoic acid, and specific degradation of the peptidoglycan into soluble fragments that are suitable for chemical analysis. Cell walls of *Bacillus stearothermophilus* B65 have been found to possess an active autolytic enzyme system that brings about their rapid dissolution (Grant & Wicken, 1968). The soluble material resulting from this autolysis process has been shown to contain a number of peptides, some at least of which are composed of alanine, glutamic acid and $\alpha\epsilon$ -diaminopimelic acid, the principal amino acid constituents of the cell wall (Forrester & Wicken, 1966). Autolysis thus provides a convenient way of obtaining soluble peptide fragments free from glycan and teichoic acid (Wicken, 1966) material. This in turn offers the possibility of determining part of the structure of the peptidoglycan as well as the points of action and specificities of the enzymes in the autolytic system. The resolution of the peptide mixture, and

analysis of the resulting homogeneous peptides with these aims in mind, form the main part of this paper.

MATERIALS AND METHODS

Growth of organisms, preparation and autolysis of cell walls. Cell walls were prepared from *B. stearothermophilus* B65 grown at 55°C as described previously (Wicken, 1966; Forrester & Wicken, 1966). Preparations other than those used for autolysis experiments were digested with trypsin and washed with water (Park & Hancock, 1960) to remove any remaining cytoplasmic protein. Autolysis was carried out as described by Grant & Wicken (1968); in the large-scale experiment, 1g of cell walls was suspended in 0.02M-sodium phosphate buffer (Gomori, 1955), pH 7.5 (50ml), and allowed to autolyse at 55°C for 3h.

Desalting. Desalting of peptides was accomplished either by gel filtration or by ion-exchange chromatography. Gel filtration was carried out on a column (45cm×2.5cm) of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) which was eluted with water at a flow rate of 15ml/h. Fractions (1ml) were collected. Peptides were located by analysis of appropriate samples (50μl) of column fractions for free amino groups (Ghuysen, Tipper

& Strominger, 1966). The ion-exchange method employed a column (20cm×1.0cm) of Dowex 50 (X8; H⁺ form) eluted first with water (120ml) and then with 0.1M-piperidine (500ml). The flow rate was approx. 30ml/h. Piperidine was removed from the desalted peptides by freeze-drying several times over conc. H₂SO₄.

DEAE-Sephadex chromatography. Peptide material was fractionated on a column (40cm×1.9cm) of DEAE-Sephadex A-50 equilibrated with 0.02M-K₂HPO₄, pH8.5. Application of the sample dissolved in 3ml of 0.02M-K₂HPO₄ was followed by elution at 18ml/h with a linear pH gradient generated by 500ml of 0.02M-K₂HPO₄, pH8.5, in the mixing vessel, and 500ml of 0.01M-citric acid-0.06M-KCl, pH2.8, in the reservoir. 170 fractions (5ml) were collected, followed by a further 115 fractions eluted with the above citric acid-KCl mixture. Appropriate samples (0.1–0.3ml) from the fractions were analysed for free amino groups by the method of Ghuyssen *et al.* (1966).

Hydrolysis conditions. Total acid hydrolysis of peptides was carried out on 30–50nmol of peptide with 6M-HCl (150μl) in sealed tubes at 100°C for 16h. The following partial acid hydrolysis conditions were used: 2M-HCl at 100°C for 3h; 4M-HCl at room temperature for 24h (Petit, Adam, Wietzerbin-Falszpan, Lederer & Ghuyssen, 1969); 10M-HCl at 37°C for 50h (slightly adapted from Pelzer, 1962). Cell walls were hydrolysed *in vacuo* with 6M-HCl at 100°C for 16h and with 4M-HCl at 100°C for 4h for analysis of amino acids and amino sugars respectively. In all cases, HCl was removed after hydrolysis by repeated evaporation from water *in vacuo* over P₂O₅ and NaOH pellets. After partial acid hydrolysis (2M-HCl, 100°C, 3h) of cell walls the hydrolysate was passed through a short column (10cm×0.7cm) of Dowex 3 (X4, free-base form) that was found to bind teichoic acid fragments. Omission of this step resulted in distortion of the paper-chromatographic properties of the peptidoglycan material in the hydrolysate.

Paper chromatography. Whatman chromatography papers nos. 1, 4 and 3MM were used. For preparative and analytical work papers were washed before use with 2M-acetic acid containing EDTA (disodium salt, 0.1%) and then with water (Wicken, 1966). The following solvent systems were used. A, butan-2-ol-formic acid-water (7:1:2, by vol.) (Roberts, Cowie, Abelson, Bolton & Britten, 1955); B, butan-1-ol-pyridine-water-acetic acid (15:10:12:3, by vol.) (Waley & Watson, 1953); C, propan-2-ol-acetic acid-water (15:2:3, by vol.) (Schleifer & Kandler, 1967); D, 2-methylpyridine-aq. ammonia (sp.gr. 0.90)-water (35:1:14, by vol.) (Schleifer & Kandler, 1967); E, ethanol-aq. ammonia (sp.gr. 0.90)-water (85:13:2, by vol.) (Tinelli, 1966); F, isobutyric acid-0.5M-ammonia (5:3, v/v) (Hughes, 1968a); G, butan-1-ol-acetic acid-water (4:1:5, by vol.), upper phase (Partridge, 1948); H, pyridine-water (4:1, v/v) (Pelzer, 1962); J, propan-1-ol-aq. ammonia (sp.gr. 0.90)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); K, butan-1-ol-pyridine-water-acetic acid (60:40:30:3, by vol.) (Primosigh, Pelzer, Maass & Weidel, 1961); L, 2-methylbutan-2-ol saturated with 0.05M-potassium hydrogen phthalate buffer (pH6.0) (Blackburn & Lowther, 1951); M, 2-methylbutan-2-ol-pyridine-acetic acid-water (110:1.0:0.08:25, by vol.) (Grant & Wicken, 1970).

All papers were developed in the descending direction

except for those in solvent J, which were ascending. Papers for system L were dipped in the phthalate buffer and dried before use.

Thin-layer chromatography. Two-dimensional t.l.c. for identification of amino acids was carried out on layers (0.25mm thick) of MN cellulose 300 (Macherey, Nagel and Co., Düren, Germany). The solvents used were slightly modified from White (1968), i.e. pyridine-acetone-aq. ammonia (sp.gr. 0.90)-water (9:6:2:3, by vol.) in the first dimension, and propan-2-ol-formic acid-water (6:1:1, by vol.) in the second. These gave an excellent and rapid separation of cell-wall amino acids and amino sugars. DNP-amino acids were identified by two-dimensional t.l.c. on layers (0.25mm thick) of a cellulose (MN300)-silica gel H (E. Merck, Darmstadt, Germany) mixture (5:2, w/w) (Grant & Wicken, 1970).

Paper electrophoresis. Low-voltage paper electrophoresis of peptides was carried out with washed Whatman no. 3MM paper at 7.3V/cm (8–10mA) in the following volatile buffer systems: pH1.8, formic acid-acetic acid-water (1:3:16, by vol.) (Matsuda, Kotani & Kato, 1968a); pH5.0, pyridine-acetic acid-water (11:8:981, by vol.); pH9.5, 0.02M-ammonium acetate, adjusted with aq. ammonia.

Determination of amino acids. Quantitative amino acid analysis of peptides was carried out as described by Forrester & Wicken (1966). Hydrolysates and standards were chromatographed on the same paper in solvent K and estimated by the cadmium acetate-ninhydrin method of Heilmann, Barollier & Watzke (1958). Amino acids in hydrolysates of whole cell walls were measured with a Beckman-Spinco amino acid analyser. Hexosamines were measured in a separate experiment. Columns were standardized with known mixtures of wall components similarly treated with acid.

Determination of N-terminal amino acids. The method of Ghuyssen *et al.* (1966) was used for 1-fluoro-2,4-dinitrobenzene treatment of the peptides, and that of Schleifer & Kandler (1967) was used on whole cell walls. The DNP-amino acids produced on subsequent hydrolysis were identified by t.l.c. as described above. Quantitative analysis was carried out by paper chromatography in solvents K (for water-soluble derivatives) and L (for ether-soluble derivatives) followed by elution of the spots with aq. 1% (w/v) NaHCO₃ and determination from the E₃₆₀ on a Shimadzu QR50 spectrophotometer (Smith, 1960; Takebe, 1965). Standard mixtures of amino acids were treated identically, thus permitting reference curves to be drawn. All operations were carried out in a darkened room.

Determination of optical configurations of amino acids. Glutamic acid (0.5mg) was isolated from a hydrolysate of cell walls by paper chromatography in solvents G and K. Its optical configuration was determined by the method of Bernt & Bergmeyer (1963). The configuration of diaminopimelic acid in peptide hydrolysates was determined by a combination of the chromatographic methods of Rhuland, Work, Denman & Hoare (1955), and Jušić, Roy, Schocher & Watson (1963).

The method of Soda (1968) was found to be generally unsatisfactory for quantitative measurement of D-alanine owing to high blank values obtained on analysis of eluates from paper chromatograms. Instead a qualitative method was employed. The peptide concerned

and control quantities of D- and L-alanine were treated with fluorodinitrobenzene, hydrolysed and extracted with ether. N-terminal alanine was isolated as its DNP-derivative by chromatography of the ether extract in solvent M, and alanine residues from the 'interior' of the peptide were obtained as the free amino acid by chromatography of the water phase in solvent K. The DNP group was removed from DNP-alanine by heating in anhydrous hydrazine at 80°C for 1 h (Heyns & Legler, 1957). Alanine from both sources and from the controls was then chromatographed in solvent J, and its configuration was determined with D-amino acid oxidase (from hog kidney, crude) (Sigma Chemical Co., St Louis, Mo., U.S.A.) by the method of Wicken & Baddiley (1963).

Other analyses. The Edman degradation procedure used was that of Tipper, Katz, Strominger & Ghuyssen (1967); hydrazinolysis was performed as described by Ghuyssen *et al.* (1966); amide ammonia was measured by an adaptation of the method of Matsuda *et al.* (1968a), with the drying step replaced by direct neutralization of excess of HCl with the calculated quantity of 4M-NaOH. Contamination by atmospheric ammonia was minimized and appropriate controls were carried out with each determination.

EXPERIMENTAL AND RESULTS

Amino acid and hexosamine analysis of cell walls. The results of analysis of whole cell walls are presented in Table 1. These compare favourably with values obtained by quantitative paper-chromatographic analysis (Forrester & Wicken, 1966), except for the detection of serine and aspartic acid in the present experiments. Amino acids accounted for 26.8% (w/w) of the cell walls. More than 99% (by weight) of the glutamic acid of the cell walls had the D configuration.

Free N-terminal groups of cells walls. Trypsin-digested walls were treated with ethanol-aq. ammonia (sp.gr. 0.90) (1:1, v/v) (Wicken & Baddiley, 1963) to remove D-alanyl ester residues

from the wall teichoic acid (Wicken, 1966). The walls were washed six times with water, treated with fluorodinitrobenzene and hydrolysed. Mono-DNP-diaminopimelic acid was the only DNP-derivative obtained. Quantitative analysis of this product (Takebe, 1965) gave a value of 12 μ mol/100 mg dry wt. of cell wall, equivalent to 30% of the total diaminopimelic acid of the walls.

Partial acid hydrolysis of whole cell walls. Trypsin-digested walls (65 mg) were partially hydrolysed with the 2M-hydrochloric acid treatment. On paper chromatography in solvents C and D the hydrolysate yielded large quantities of alanine, glutamic acid and diaminopimelic acid, small amounts of muramic acid, glucosamine, glycine and serine, and three major and several minor peptides. The minor peptides were not examined further, but the major peptides were purified by paper chromatography in solvents B, C, D and E and analysed. The chromatographic mobilities and the analytical results are shown in Table 2.

The results show that peptide I was the dipeptide Ala-Dap,* peptide II was its isomer Dap-Ala, and peptide III was the tripeptide Dap-Ala-Dap.

Autolysis and fractionation of the soluble autolysate. Cell walls (1 g) were autolysed and, after removal of the insoluble residue (33 mg) by centrifugation, the soluble fraction (autolysate) was extracted three times with ether (reflux conditions 40°C) and three times with chloroform-methanol (2:1, v/v) (reflux conditions, 60–70°C). The total extracted lipid amounted to 1.5 mg. The extracted autolysate was dialysed against three changes of water (21) at 4°C. The non-diffusible portion accounted for 64% by weight of the original walls, and was composed of a peptidoglycan-teichoic acid complex (Grant &

* Abbreviation: Dap (in tables and amino acid sequences), α -diaminopimelic acid residue.

Table 1. *Analysis of amino acids and hexosamines from cell walls of B. stearothermophilus B 65*

Results refer to dry weight of cell walls. Hydrolysis conditions were 4M-HCl at 100°C for 4 h (hexosamines) and 6M-HCl at 100°C for 16 h (amino acids). Analysis was carried out on a Beckman-Spinco amino acid analyser. Molar ratios are calculated relative to glutamic acid.

Compound	Composition (μ mol/100mg)	Composition (%, w/w)	Molar ratios
Alanine	108	9.6	2.30
Diaminopimelic acid	40	7.6	0.85
Glutamic acid	47	6.9	1.00
Aspartic acid	5	0.7	0.11
Glycine	6	0.5	0.13
Serine	14	1.5	0.31
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	Sum	26.8	
Glucosamine	30	5.0	0.64
Muramic acid	28	7.5	0.60

Table 2. *Properties of peptides obtained on partial acid hydrolysis of whole cell walls of B. stearothermophilus*

Cell walls were hydrolysed with 2M-HCl at 100°C for 3h. Peptides were isolated by paper chromatography of the hydrolysate. Compositions were determined by analytical paper chromatography. *N*-terminals were identified by fluorodinitrobenzene treatment, and *C*-terminals by hydrazinolysis. Chromatographic results are expressed relative to diaminopimelic acid in solvents C and D. Molar ratios are calculated relative to alanine (=1.00). N.D., Not determined.

Peptide	R_{Dap} (solvent C)	R_{Dap} (solvent D)	Composition (molar ratios)		<i>N</i> -Terminal amino acids			<i>C</i> -Terminal amino acids	
					Mono-amino-		Di-amino-	Ala	Dap
			Ala	Dap	Ala	Dap	Dap		
I	1.67	1.62	1.00	1.02	+	+	—	—	+
II	2.3	1.67	1.00	1.04	—	—	+	N.D.	
III	0.29	0.42	1.00	1.93	—	+	+	—	+

Fig. 1. Separation of the peptide mixture from the diffusible fraction of the autolysate of cell walls of *B. stearothermophilus* on a column (40cm×1.9cm) of DEAE-Sephadex A-50. The starting buffer was 0.02M- K_2HPO_4 , pH8.5, and a linear pH gradient (----) was used to elute the column at a rate of 18ml/h; 5ml fractions were collected. After 850ml had been collected the gradient was replaced with 0.01M-citric acid-0.06M-KCl, pH2.8. A total of 285 fractions was collected. Fluorodinitrobenzene analysis was performed without prior hydrolysis on samples (0.1–0.3ml) of every second fraction. No significant peaks were detected after fraction 245.

Wicken, 1968) and a small quantity of peptidoglycan. The diffusate, after rotary evaporation to a small volume, was desalted on Dowex 50 resin. Paper chromatography of this material in solvent A showed that it contained a large quantity of alanine, small quantities of serine and glycine, traces of glutamic and diaminopimelic acids, and a mixture of peptides, none of which contained hexosamine

residues. The alanine was shown to have the D configuration. The gross composition of the diffusate was thus entirely amino acid in nature, it accounted for approx. 21% by weight of the dry cell walls, and hence about 80% of the total amino acids present in these walls (see Table 1).

The peptide mixture of the diffusate was separated from the free amino acids by preparative

chromatography in solvent A. DEAE-Sephadex chromatography of this mixture resulted in the elution profile shown in Fig. 1. Fractions B to F were decreased in volume by rotary evaporation and desalted on a column of Sephadex G-10. Fraction A could not be separated from salt on Sephadex G-10 and a mixture of small fragments of very low yield was obtained from this peak after desalting on Dowex 50. These were not examined further. Paper chromatography in solvent B separated the desalted fractions B, D and F into two components each (B_1 , B_2 , D_1 , D_2 , F_1 and F_2), fraction C into three components (C_1 , C_2 and C_3) but did not resolve fraction E any further. Paper electrophoresis at pH values of 1.8, 5.0 and 9.5 separated fractions C_2 , E and F_1 respectively into

a major and a minor component, but the yields of the minor components were too low to permit any further investigation of their properties.

The homogeneity of the ten major peptides was further established by paper chromatography in solvents B, F, G and H, and by paper electrophoresis at pH 1.8 and pH 5.0. Their chromatographic and electrophoretic mobilities are shown in Table 3.

Analysis of the autolysate peptides

Amino acid composition, *N*-terminal residues and amide-ammonia content of the peptides were determined. All were composed of alanine, glutamic acid and diaminopimelic acid, all possessed *N*-terminal alanine and mono- or di-*N*-terminal

Table 3. *Chromatographic and electrophoretic properties of the major peptides from the autolysate of cell walls of B. stearothermophilus*

The chromatographic mobilities are given relative to diaminopimelic acid in the solvents shown. The electrophoretic migrations are given relative to lysine and are corrected for endosmotic flow; the sign indicates the charge on the peptide. Solvents and buffers are given in the Materials and Methods section. N.D., Not determined.

Peptide	Chromatography R_{Dap} in solvents				Electrophoresis M_{Lys}	
	B	F	G	H	pH 1.8	pH 5.0
B_1	0.58	1.36	0.16	2.0	+0.76	+0.44
B_2	0.81	1.13	0.77	1.6	+0.66	+0.05
C_1	0.36	1.07	0.15	1.0	+0.63	+0.05
C_2	0.48	1.11	0.24	1.2	+0.63	+0.03
C_3	0.71	0.88	0.90	1.0	+0.57	-0.53
D_1	0.27	0.83	0.18	0.0	+0.57	-0.25
D_2	0.41	0.89	0.33	0.0	+0.62	-0.27
E	0.29	0.89	0.18	0.0	+0.62	-0.17
F_1	0.11	0.80	0.00	0.0	+0.61	-0.17
F_2	0.25	N.D.	N.D.	N.D.	N.D.	-0.54

Table 4. *Analysis of the major peptides from the autolysate of B. stearothermophilus cell walls*

The composition and *N*-terminals are expressed as molar ratios, relative to 1, 2, or 3 mol of glutamic acid. The peptides are set out in four groups corresponding to the four different structures proposed in the text. N.D., Not determined.

Peptide	Composition (molar ratios expressed relative to glutamic acid)						
	Ala	Glu	Dap	NH ₃	<i>N</i> -Terminal Ala	Mono- <i>N</i> -terminal Dap	Di- <i>N</i> -terminal Dap
B_2	1.02	1.00	1.04	1.03	1.02	1.11	Nil
C_3	1.09	1.00	1.03	0.4*	1.08	0.97	Nil
B_1	2.06	1.00	1.82	1.98	0.92	Trace	Present†
C_2	1.95	1.00	2.24	0.93	1.11	Nil	0.98
D_2	2.06	1.00	2.04	0.6*	1.04	Nil	1.01
C_1	2.86	2.00	2.10	2.86	2.14	0.95	Nil
D_1	3.14	2.00	2.20	1.12	2.22	1.05	Nil
E	2.98	2.00	1.96	1.28	2.22	0.98	Nil
F_2	2.94	2.00	2.06	N.D.	1.98	1.00	Nil
F_1	5.22	2.00	3.27	2.01	3.36	0.77	Nil

* High ammonia content of the peptide preparation. (Determination less reliable.)

† Di-DNP-diaminopimelic acid not determined quantitatively.

diaminopimelic acid, and most were amidated to some extent. The analysis results are presented in Table 4.

There were thus two tripeptides (B_2 and C_3), three pentapeptides (B_1 , C_2 and D_2), four heptapeptides (C_1 , D_1 , E and F_2) and a possible undecapeptide (F_1). The tripeptide B_2 was quantitatively the most important peptide, even allowing for known losses of peptides C_3 , D_2 and E during purification. The configuration of diaminopimelic acid was determined in all peptides except E and F_1 , and was found to be *meso* in every case.

Tripeptides B_2 and C_3 . The alanine residue (*N*-terminal) was shown to have the *L* configuration in both peptides, and after one cycle of Edman

degradation it was replaced by *N*-terminal glutamic acid, as shown by dinitrophenylation. Most of the mono-*N*-terminal diaminopimelic acid disappeared simultaneously as a result of substitution by the phenylthiocarbamyl group (Kato, Strominger & Kotani, 1968; van Heijenoort *et al.* 1969). After a second cycle of degradation, both peptides lost their *N*-terminal glutamic acid without release of *N*-terminal diaminopimelic acid, suggesting that the γ -carboxyl group of the glutamic acid was involved in the peptide bond to the diaminopimelic acid residue (cf. Hungerer, Fleck & Tipper, 1969).

At pH 5.0 a tripeptide Ala-Glu-Dap would be negatively charged. Peptide C_3 was negatively charged at this pH (Table 3) and it is therefore

Fig. 2. Proposed structures of the peptides from the autolysate of *B. stearothermophilus* cell walls. The diagrams show the non-animated peptide structures.

likely that C₃ was the above simple tripeptide. Hydrazinolysis of peptide C₃ released 104 nmol of free diaminopimelic acid/100 nmol of peptide, thus confirming the proposed sequence.

The amide-ammonia content of peptide B₂ and its electrical neutrality on electrophoresis at pH 5.0 indicated that it was a monoamide of the peptide C₃ structure. Hydrazinolysis of peptide B₂ released no free amino acid, which implies that the amide substituent was on one of the free carboxyl groups of the diaminopimelic acid residue (Kato *et al.* 1968). The tripeptide structure of B₂ and C₃ is shown in Fig. 2(a).

Pentapeptides B₁, C₂ and D₂. The *N*-terminal residue was L-alanine in these three peptides, and the 'internal' alanine residue had the D configuration in each case.

A single cycle of Edman degradation of peptide C₂ yielded a residual peptide. Fluorodinitrobenzene treatment of this peptide followed by acid hydrolysis and quantitative analysis of the products gave the results shown in Table 5. These are consistent with the sequence N^α-glutamyl-(N^ε-alanyl)-di-

aminopimelic acid. At pH 5.0 this structure would possess an extra negative charge. However, the peptide did not migrate on electrophoresis at pH 5.0, which means that it was probably a monoamide. The release of this peptide by Edman degradation is strong evidence for peptide C₂ having the type of structure shown in Fig. 2(b).

Insufficient material was available for Edman degradations to be carried out on peptides B₁ or D₂, but it is suggested that they were differently amidated forms of the structure shown in Fig. 2(b). Peptide C₂ was almost certainly a monoamide, as shown by its ammonia content and its electrophoretic mobility at pH 5.0. The conversion of the amidated carboxyl group after Edman degradation indicates that it belonged either to the 'internal' diaminopimelic acid residue or to the glutamic acid. Peptide B₁ was highly positively charged at pH 5.0, which would be expected from its high amide ammonia content and it was probably a diamide. The negative charge on peptide D₂ at pH 5.0 indicates that it was probably free of amide ammonia.

Heptapeptides C₁, D₁, E and F₂. Both *N*-terminal alanine residues in peptides C₁, D₁ and F₂ had the L configuration, and the 'internal' residue was D-alanine in these three peptides.

One cycle of Edman degradation of peptides C₁ and D₁ resulted in the loss of all *N*-terminal alanine and almost all of the *N*-terminal diaminopimelic acid. The alanine/glutamic acid ratio was 1.10:2.00 in the resulting residual peptides, and 80–90% of the glutamic acid was shown to have a free *N*-terminal group. A second cycle of degradation resulted in the loss of the glutamic acid residues without the appearance of any new *N*-terminal groups, again suggesting the involvement of the γ-carboxyl group of glutamic acid in the peptide

Table 5. Quantitative analysis of a peptide released from peptide C₂ after one cycle of Edman degradation

The peptide was dinitrophenylated and hydrolysed as described in the Materials and Methods section. Separation and determination of DNP-amino acids was followed by chromatography and analysis of the water phase by the cadmium acetate-ninhydrin procedure.

	Free Dap	DNP-alanine	DNP-glutamic acid
Content (nmol)	74.0	84.3	89.9
Molar ratios (DNP-Glu = 1.00)	0.82	0.94	1.00

Table 6. Characteristics and analysis of peptides obtained by partial acid hydrolysis of peptide F₂

Hydrolysis at 100°C for 3 h in 2M-HCl yielded peptides 1–4, and treatment with 10M-HCl for 50 h at 37°C yielded a mixture from which peptides 5, 6 and 7 were separated. Compositions are given as molar ratios relative to alanine or glutamic acid (1.00). Chromatographic mobilities are expressed relative to diaminopimelic acid in solvents B, C, D and F. Electrophoretic migrations are expressed relative to lysine at pH 5.0. N.D., Not determined.

Peptide	R_{Dap} in solvents				M_{Lys} pH5.0	Composition (molar ratios)			<i>N</i> -Terminal amino acids		
	B	C	D	F		Ala	Glu	Dap	Ala	Mono-amino- Dap	Di-amino- Dap
1			2.25			1.00	1.00	nil	+	—	—
2		1.68				1.00	0.24	0.77	+	+	—
3		2.31		1.15		1.00	0.17	0.91		N.D.	
4		0.28	0.42			1.00	0.15	1.58		N.D.	
5			2.20			1.00	0.99	Nil	+	—	—
6	0.72			0.90	−0.42	1.07	1.00	0.99	+	+	—
7	0.72			0.82	−0.52	0.13	1.00	0.95		N.D.	

linkage to diaminopimelic acid. These results indicate that peptides C₁ and D₁ had the same basic structure, as shown in Fig. 2(c). Table 4 shows that peptide C₁ contained 2.86 mol of ammonia/mol of peptide but its electrical neutrality (Table 3) at pH 5.0 would suggest a diamide rather than a triamide form. Peptide D₁ was almost certainly a monoamide (Tables 3 and 4) from its ammonia content and electrophoretic properties.

Peptide E has not been examined as fully as the other heptapeptides because of loss of material owing to difficulties in its purification. In ammonia content, electrophoretic and chromatographic properties it resembled peptide D₁, and it is possible that these two peptides were isomeric in that the single amide substituent may have been on different carboxyl groups.

Peptide F₂, the fourth heptapeptide, was highly negatively charged at pH 5.0. It was therefore probably non-amidated, although its ammonia content was not determined. In support of this conclusion is the fact that partial acid hydrolysis of peptides C₁ and D₁ under mild conditions (4 M-hydrochloric acid at room temperature for 24 h) yielded a mixture of products, a major one of which from both hydrolysates migrated with peptide F₂ on electrophoresis at pH 5.0. No species migrating towards the cathode at a greater rate was detected.

The structure proposed for the heptapeptides (Fig. 2c) is supported by a series of peptide fragments obtained from partial acid hydrolysis of peptide F₂, by using the 2 M- and 10 M-hydrochloric acid treatments. Paper chromatography of the hydrolysates in solvents B, C, D and F, and paper electrophoresis at pH 5.0, yielded four peptides from the 2 M-hydrochloric acid treatment and three from the 10 M-hydrochloric acid treatment as well as alanine, glutamic acid and diaminopimelic acid. The peptides were analysed and the results are presented in Table 6.

Peptides 1 and 5 were identical. The alanine residue was shown to have the L configuration, and the structure was therefore L-Ala-Glu. Peptides 2, 3 and 4, though not fully purified and characterized, were directly comparable, in composition, N-terminal analysis and chromatographic behaviour, with peptides I, II and III respectively from the partial acid hydrolysis of whole cell walls (see Table 2). It is probable, therefore, that peptides 2, 3 and 4 were Ala-Dap, Dap-Ala and Dap-Ala-Dap respectively. Peptide 6 had the partial sequence Ala-(Glu, Dap) and closely resembled the autolysis peptide C₃ in its chromatographic and electrophoretic mobilities (cf. Table 3). It was therefore almost certainly Ala-Glu-Dap. Finally, peptide 7 was the dipeptide (Glu, Dap). Its sequence was not determined owing to lack of sufficient material.

Two-dimensional t.l.c. in solvents C and D of

partial acid hydrolysates of peptides C₁ and D₁ gave patterns strikingly similar to those obtained from peptide F₂ by the same treatments.

Peptide F₁. The low proportions of mono-N-terminal diaminopimelic acid in this peptide, and its relatively low chromatographic mobilities in solvents B, F, G and H indicate that this peptide was probably of higher molecular weight than those described above. Molar ratios of component amino acids and N-terminal analysis are consistent with a diamide of the undecapeptide structure proposed in Fig. 2(d).

DISCUSSION

Autolysis of bacterial cell walls generally occurs through enzymic hydrolysis of specific linkages in the peptidoglycan. In *Streptococcus faecalis* (Shockman, Thompson & Conover, 1967) and *Arthrobacter crystallopoietes* (Krulwich & Ensign, 1968) the glycan chains are cleaved by *endo*-N-acetylmuramidases, and in *Bacillus subtilis* (Young, 1966a,b), *Staphylococcus aureus* (Tipper, 1969) and *Listeria monocytogenes* (Tinelli, 1969) the glycan and peptide moieties of the heteropolymer are separated by the action of an N-acetylmuramyl-L-alanine amidase. Several bacteriolytic enzymes from other sources possess these activities (Ghuysen *et al.* 1966; Ghuysen, 1968), and both types occur in the autolytic systems of *Escherichia coli* B (Pelzer, 1963; Weidel & Pelzer, 1964) and *B. thuringiensis* var. *thuringiensis* (Kingan & Ensign, 1968).

The structures of the ten major peptides released on autolysis of walls of *B. stearotheophilus* B 65 (Figs. 2a-d) are consistent with the principal autolysin in these walls being an N-acylmuramyl-L-alanine amidase. The absence of peptides linked to small glycan fragments, the lack of diffusible oligosaccharides in the autolysate and the lack of an increase in the reducing power of autolysing wall suspensions (Grant & Wicken, 1968) indicate that little or no *endo*-N-acylmuramidase activity occurred.

The structures of relatively few diaminopimelic acid-containing peptidoglycans have been thoroughly investigated since the work on the rigid layer of the cell wall of *E. coli* B by Weidel & Pelzer (1964). Kato *et al.* (1968) and Matsuda *et al.* (1968a,b) have shown that the basic structures of the peptidoglycans from *Corynebacterium diphtheriae* and *Lactobacillus plantarum*, respectively, closely resemble the *E. coli* type, i.e. tetrapeptides (L-Ala-D-Glu[±]-meso-Dap-D-Ala) linked to glycan chains by N-acetylmuramyl-L-alanine bonds, and cross-linked to one another by peptide bonds between the carboxyl groups of the C-terminal D-alanine residues and the free amino groups of the meso-diaminopimelic acid residues (cf. van Heijenoort *et al.* 1969). Evidence has been obtained for a similar type of

structure in the peptidoglycans of *L. inulinus* (Weiss, Plapp & Kandler, 1967), *B. licheniformis* (Hughes, 1968a,b; Mirelman & Sharon, 1968), *B. subtilis* (Warth & Strominger, 1968) and *B. megaterium* (van Heijenoort *et al.* 1969), although some of the cross-linkages in this last organism involve D,D-diaminopimelic acid residues, and their exact nature is still obscure.

The structures of the tri- and hepta-peptides (Figs. 2a and 2c), quantitatively the most important peptides in the autolysate of *B. stearothermophilus* cell walls, show that the peptide network of the peptidoglycan is also very similar to the classical *E. coli* type, with direct cross-linking between D-alanine and diaminopimelic acid residues. Further evidence for this linkage is provided by the determination of the structure of the pentapeptide C₂ (Fig. 2b) and by the isolation of the peptides Ala-Dap, Dap-Ala and Dap-Ala-Dap from a partial acid hydrolysate of whole cell walls.

Dinitrophenylation of whole walls showed that only 30% of the total diaminopimelic acid residues possessed a free amino group, implying that the intact peptidoglycan is probably highly cross-linked. Such a structure would necessarily involve a large proportion of undecapeptide (Fig. 2d) or even larger units, and the finding that the quantitatively predominant species in the autolysate were the tri- and hepta-peptides suggests that lysis of cross-linkages may have occurred during the autolysis process. An endopeptidase catalysing the hydrolysis of N^ε-(D-alanyl)-*meso*-diaminopimelic acid cross-linkages is present in the *E. coli* autolytic complex (Pelzer, 1963) and in the L3 and KM enzyme preparations from *Streptomyces* species (Matsuda, Kotani, Kato, Moriyama & Yoneda, 1968; Ghuyssen *et al.* 1969). The existence of a similar enzyme in walls of bacilli is suggested by the observation by Dadd & Paulton (1968) that free amino groups of diaminopimelic acid residues in walls prepared from synchronized cultures of *B. subtilis* increased by approx. 30% between cell divisions. It is therefore likely that 'extent' of cross-linking is an unreal concept in that it is determined at any given time by a balance of various activities of degradative and biosynthetic enzymes in the cell wall.

The presence of pentapeptides of the type shown in Fig. 2(b) in autolysates of these walls suggests an endopeptidase activity directed towards the D-glutamyl-*meso*-diaminopimelic acid linkage. Although such an activity has not been reported as occurring in other autolytic systems, identical pentapeptides have been isolated from autolysates of walls of *B. subtilis* (A. D. Warth, personal communication).

All of the major peptides obtained on autolysis were completely lacking in C-terminal D-alanine

residues, a feature that has been noted in peptides obtained from diaminopimelic acid-containing peptidoglycans of other Gram-positive bacteria. It seems likely that D-alanine carboxypeptidases of the type found in the *E. coli* autolytic complex (Pelzer, 1963; Leutgeb & Weidel, 1963) may be responsible, but any activity *in vitro* of such an enzyme in autolysing *B. stearothermophilus* cell walls was difficult to detect because of the accompanying hydrolysis of the labile D-alanyl ester substituents of the teichoic acid of these walls (Wicken, 1966) under the mildly alkaline conditions employed (cf. Mirelman & Sharon, 1968).

The range of degree of amidation of the autolysate peptides reported here resembles that found in peptides from *C. diphtheriae* (Kato *et al.* 1968), *L. plantarum* (Matsuda *et al.* 1968a,b), *B. subtilis* (Warth & Strominger, 1968; A. D. Warth, personal communication) and *Mycobacterium smegmatis* (Petit *et al.* 1969). It is possible that this reflects different degrees of deamidation of fully- or almost fully-amidated structures by an enzyme similar to that present in the *Streptomyces* L3 enzyme preparation (Kato *et al.* 1968).

Finally, the lack of peptides containing residues of aspartic acid, glycine or serine should be noted. These amino acids are often found as minor components of hydrolysates of Gram-positive cell walls, but their role in wall structure is still unknown.

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