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CHEMICAL STRUCTURE OF THE PEPTIDOGLYCAN OF *VIBRIO PARAHAEMOLYTICUS* A55 WITH SPECIAL REFERENCE TO THE EXTENT OF INTERPEPTIDE CROSS-LINKING[1]

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SUMMARY   The chemical structure of the cell wall peptidoglycan of *Vibrio parahaemolyticus* A55 was studied.   Estimation of cross linkages between peptide subunits in the peptidoglycan by dinitrophenylation showed that about 30% of the total 2,6-diaminopimelic acid (A_2pm) residues were involved in cross linkages.   The presence of interpeptide bridges was also demonstrated by isolating bisdisaccharide peptide subunit dimers from *Chalaropsis* muramidase digests of the cell wall peptidoglycan by gel filtration followed by ion-exchange column chromatography, although most of the building blocks obtained were uncross-linked disaccharide peptide monomers.   The chain length of a glycan moiety of the peptidoglycan obtained by treatment with the L-11 enzyme and gel filtration of the digest was also studied.   The chain length varied from 7 to 44, but 30% of the glycan fragments had muramic acid at the reducing end and a chain length of 28 to 44.   In conformity with the above structural study it was demonstrated that a particulate enzyme fraction obtained by differential centrifugation of a sonicated preparation of *V. parahaemolyticus* catalyzed a penicillin-sensitive transpeptidation reaction, using UDP-MurNAc-^{14}C-pentapeptide and UDP-GlcNAc as substrates.

INTRODUCTION

A preceding paper (Tamura et al., 1976) described the isolation of a bag-shaped cell wall peptidoglycan (murein sacculus) from *V. parahaemolyticus* A55 by treatment of whole cell with sodium dodecylsulfate (SDS) and then digestion with protease.   The amino acid and amino sugar compositions of the isolated peptidoglycan of this halophilic bacterium were

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1 Parts of this study were read at the 44th and 46th Annual Meetings of the Japanese Bacteriological Society in Tokyo, in April 1971 and 1973.

similar to those of non-halophilic gram-negative bacteria.

This paper reports studies on the structure of the *V. parahaemolyticus* peptidoglycan with special reference to the extent of cross-linkages between peptide subunits. Studies were made in two ways: 1) the building blocks of peptidoglycan obtained by enzymic digestion were isolated and their costituent, N-terminal and C-terminal amino acids were analyzed quantitatively, and 2) the formation of interpeptide cross linkages by a particulate enzyme of *V. parahaemolyticus* was examined using UDP-*N*-acetylmuramyl-^{14}C-pentapeptide (see later) and UDP-*N*-acetylglucosamine as substrates, and the inhibitory effect of penicillin on this reaction was examined.

MATERIALS AND METHODS

1. *Bacterial strains*

V. parahaemolyticus A55 (serotype, 05: K15) was maintained as described in a previous paper (Tamura et al., 1976). *Pseudomonas* sp. 101 used as a reference strain was supplied by courtesy of Dr. M. Masui (Department of Microbiology, Osaka City University School of Medicine).

2. *Chemicals and enzymes*

Uridine 5'-diphosphate-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-2,6-diaminopimelyl-D-[^{14}C]-alanyl-D-[^{14}C]alanine (UDP-MurNAc-^{14}C-pentapeptide) was a gift from Dr. K. Izaki (Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai) and had a specific activity of 66 μCi per μmole. UDP-*N*-acetylglucosamine (UDP-GlcNAc) was purchased from Sigma Pure Chemicals Co. Ltd. (St. Lois, Mo., USA) and penicillin G was supplied by Meiji Seika Co. Ltd. (Tokyo). The specimens of glucosaminitol and muramicitol used as references were kindly supplied by Drs. K. Yamamoto and S. Hara, respectively, Department of Chemistry, Osaka University Faculty of Science.

Chalaropsis enzyme was a gift from Dr. I. Ringler (Lederle Labs., Div. American Cyanamide Co., Pearl River, N.Y., USA), and *Flavobacterium* L-11 enzyme (crude) was supplied from Kyowa Fermentation Industry. D-Amino acid oxidase was prepared from pig kidney by the method of Massey et al. (1961) and L-glutamic acid decarboxylase was purchased from Wako Pure Chemicals Co. (Osaka).

3. *Preparation of peptidoglycan from V. parahaemolyticus*

The peptidoglycan specimen used in this study was a preparation at purification step 3 described in a previous paper (Tamura et al., 1976). The preparation consisted of bag-shaped structure of the insoluble basal layer of *V. parahaemolyticus* cell walls isolated by SDS-treatment and purified by protease digestion.

4. *Analytical methods*

Amino sugars and amino acids of the peptidoglycan and its enzymic digests were determined in a Hitachi amino acid analyzer (Model KLA-3B, Hitachi Ltd. Co., Tokyo) after hydrolysis in 6 N hydrochloric acid at 100 C for 14 hr. N-Terminal amino acids were determined from the difference in amino acid contents of the test sample and after dinitrophenylation. C-Terminal amino acids were released by hydrazinolysis of the test specimen in anhydrous hydrazine at 100 C for 6 hr and measured with an amino acid analyzer.

Free amino groups and total amino groups were determined by the DNP method (Ghuysen et al., 1966). For determination of total amino groups samples were hydrolyzed in 6 N hydrochloric acid at 100 C for 14 hr in sealed tubes and then dried *in vacuo* over sodium hydroxide. Reducing sugars, were determined by the ferricyanide method (Park and Johnson, 1942). Hexosamines and hexoses were determined by the Morgan-Elson method and the anthrone method described by Ashwell (1957) and Ghuysen et al. (1966), respectively. Isomers of A$_2$pm in the peptidoglycan or peptide subunits were determined after dinitrophnylation of their acid hydrolysate by chromatography on thin layer plate (silica gel G acc. to Stahl, type 60, Merck) in a solvent of benzyl alcohol-chloroform-methyl alcohol-water-15 N ammonia (30: 30: 30: 6: 2, v/v) as described by Bricas et al. (1967).

5. *Determination of the mean chain length of the glycan moiety of the test peptidoglycan after enzymic digest*

An appropriate amount of test specimen containing about 200 nmoles of amino sugars was dried and mixed with 600 μliters of ice cold 0.1 M sodium borohydride. The reaction mixture was stood for

16 hr at room temperature. Then it was neutralized with 300 μliter of 0.2 M acetic acid, dried *in vacuo* over concentrated sulfuric acid and hydrolyzed in 6 N hydrochloric acid at 100 C for 14 hr. The contents of aminosugar alcohols formed by borohydride reduction of the amino sugars at the reducing end and of remaining amino sugars were determined with the amino acid analyzer. The mean chain length was calculated from the equation,

$$\frac{glucosamine + glucosaminitol}{glucosaminitol} \times 2$$

or
$$\frac{muramic\ acid + muramicitol}{muramicitol} \times 2$$

6. *Assay for peptidoglycan synthesis*

The method used was in principal the same as that described previously (Suginaka et al., 1974). The particulate fraction derived from the cytoplasmic membranes was prepared from cells in cultures of either *V. parahaemolyticus* A55 or *Pseudomonas* sp. 101, at the time of half-maximal growth, by sonication with a Super Sonic Vibrator, Model UR-150 (Tominaga Works, Ltd. Co., Tokyo) and then differential centrifugation. The incubation mixture in a total volume of 25 μliters, containing 0.26 mg of protein of particulate fraction, 5 μmoles of tris(hydroxymethyl)aminoethane-hydrochloride buffer, pH 7.5, 1 μmole of magnesium chloride, 0.92 nmoles of the UDP-MurNAc-^{14}C-pentapeptide described above and 9 nmoles of UDP-GlcNAc, with or without 5 μg of penicillin G, was incubated at 37 C for 2 hr. Then the mixture was used for assay of formation of peptidoglycan, alanine and lipid intermediates, as described previously (Suginaka et al., 1974).

RESULTS

1. *Isolation and identification of the principal building blocks of V. parahaemolyticus cell wall peptidoglycan*

1) Fractionation of the *Chalaropsis* enzyme digest of *V. parahaemolyticus* peptidoglycan by gel filtration and chromatography on Dowex-1

One gram of a peptidoglycan preparation (step 3, Tamura et al., 1976) was digested with 1 mg of the *Chalaropsis* enzyme in 50 ml of 0.025 M acetate buffer, pH 4.5 for 24 hr

at 37 C. The reaction mixture was then centrifuged at 13,000×g for 15 min to remove contaminating poly-β-hydroxybutyrate and the supernatant was applied to a Sepharose 2B column (3.0×100 cm). The column was eluted with water. The material was separated into a high molecular weight fraction containing about 90% of the hexose in the test peptidoglycan, which was eluted in the void volume, and low molecular weight fraction containing essentially all of the amino acids and amino sugars with about 10% of the hexose (Fig. 1).
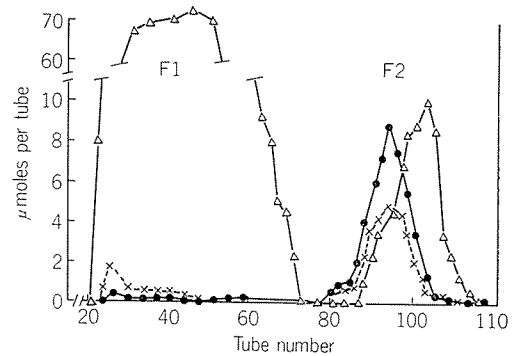

FIGURE 1. Gel filtration of the *Chalaropsis* enzyme digest of the *V. parahaemolyticus* peptidoglycan on a Sepharose 2B column. Fractions of 5 ml of eluate were collected and assayed for total amino groups (●——●), amino sugars (×——×) and hexose (△——△).

The low molecular weight fraction (tube Nos. 70 to 100, F2 in Fig. 1) was concentrated and further fractionated on a Sephadex G-75 column (2.5×100 cm). As shown in Fig. 2, a high molecular weight fraction (F2-1) consisting entirely of hexose, a lower molecular weight fraction (F2-2) containing a considerable amount of hexose, amino acids and amino sugars, and a third fraction (F2-3) detected by measuring free and total amino groups were obtained. The lower molecular weight fraction, F2-2, was concentrated and then submitted to gel filtration on Sephadex G–50, G–50 and G–25 columns connected in series. The contents of tube Nos. 44–74
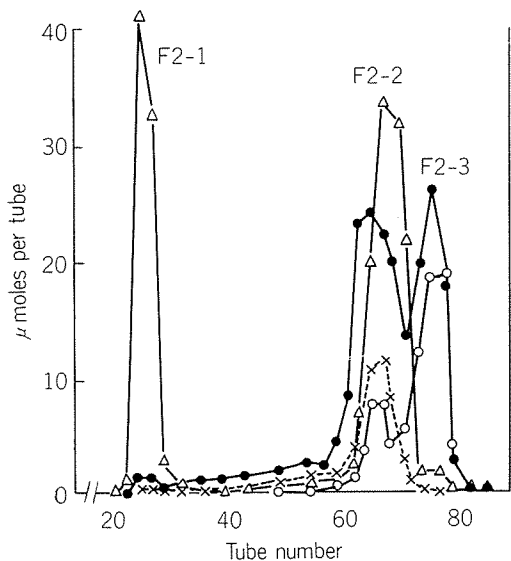
FIGURE 2. Gel filtration of the lower molecular weight fraction (F2 in Fig. 1) of the *Chalaropsis* enzyme digest on a Sephadex G-75 column. Fractions of 5 ml of eluate were collected and assayed for total amino groups (●——●), free amino groups (○——○), amino sugars (×——×) and hexose (△——△).
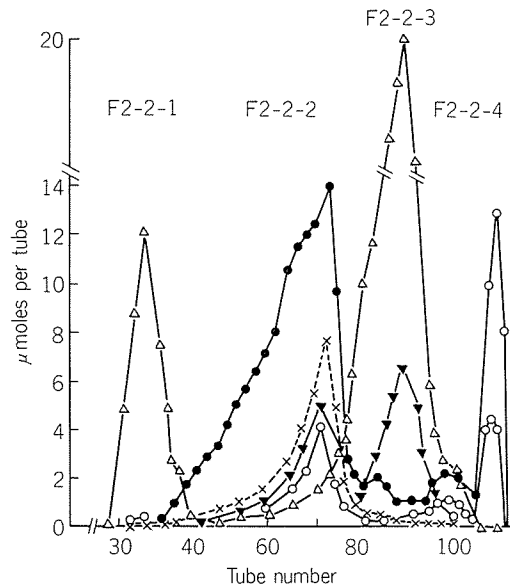

FIGURE 3. Gel filtration of fraction F2-2 (in Fig. 2) obtained from the *Chalaropsis* enzyme digest using serial columns of Sephadex G-50, G-50 and G-25. Fractions of 7 ml of eluate were collected and assayed for total amino groups (●——●), free amino groups (○——○), amino sugars (×——×), reducing sugars (▼——▼) and hexose (△——△).

(F2-2-2 in Fig. 3) which contained practically all the total amino groups and amino sugars in the specimens but no significant amount of material containing hexose were combined, concentrated and further fractionated by ion-exchange chromatography on a Dowex-1×8 (acetate form, 200–400 mesh) column (2.5× 23 cm). Seven peak fractions (P-1 to P-7) were found by determination of total amino groups (Fig. 4). Analyses of the amino acid and amino sugar compositions of these peak fractions (Table 1) showed that all the fractions except P-1 contained muramic acid, glucosamine, glutamic acid, alanine and A₂pm. Additional amino acids were found in P-2 and P-3.

The main peak fraction, P-4 containing nearly equimolar amounts of muramic acid, glucosamine, glutamic acid, A₂pm and ammonia and double this amount of alanine, was further purified and submitted to terminal amino acid
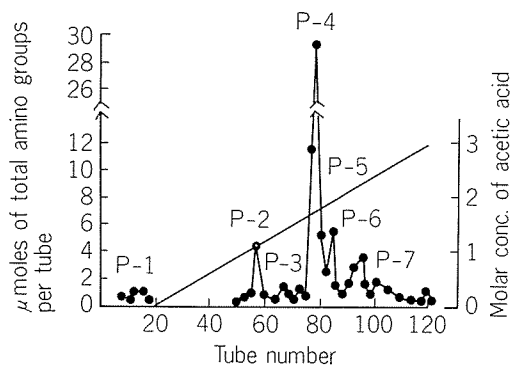

FIGURE 4. Fractionation of F2-2-2 (in Fig. 3) of the *Chalaropsis* enzyme digest on a Dowex-1×8 column (acetate form). Fractions of 10 ml of eluate were collected and assayed for total amino groups (●——●).

and amino sugar analyses as follows.

2) Rechromatography and chemical analyses of P-4 fraction

TABLE 1. *Amino acid and amino sugar compositions of the fractions from the Dowex-1 column[a]*

Constituent	P-2	P-3	P-4	P-5	P-6	P-7
M-6-P[b]	0.03	0.06	—	0.06	0.05	0.05
Asp	0.12	0.21	—	—	—	—
Thr	0.16	0.06	—	—	—	—
Ser	0.06	—	—	—	—	—
Mur[c]	0.38	0.72	0.82	0.19	0.74	0.52
Glu	1.00	1.00	1.00	1.00	1.00	1.00
Gly	0.18	—	—	—	—	—
Ala	1.63	1.79	1.98	1.88	1.83	1.84
A_2pm	0.86	0.87	0.98	0.88	0.87	1.32
GlcN	0.37	0.87	0.80	0.19	0.69	0.55
Lys	0.57	0.45	—	—	—	—
NH_3	1.33	0.60	0.73	0.50	0.80	1.03

[a] Values are moles per mole of total glutamic acid residues.
[b] M-6-P: muramic acid 6-phosphate.
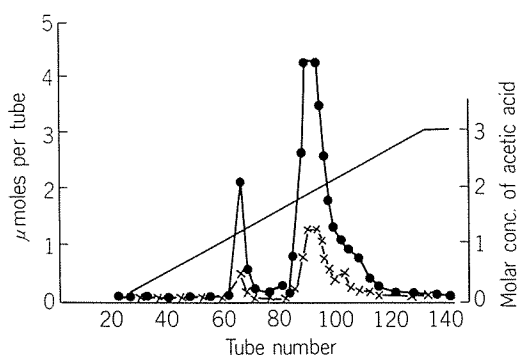[c] The value for muramic acid was corrected for loss during acid hydrolysis.

FIGURE 5. Rechromatography of P-4 on a Dowex-1 × 8 column (acetate form). Fractions of 5 ml of eluate were collected and assayed for total amino groups (●———●) and amino sugars (×———×).

One ml of P-4 fraction, containing 576 nmole equivalents of glutamic acid, was applied to a column (2.5 × 25.5 cm) of Dowex-1 × 8 (acetate form, 200–400 mesh). Elution was carried out first with 100 ml of water and then with a linear gradient of 0 to 3 M acetic acid. As shown in Fig. 5, one main peak fraction was eluted with about 2 M acetic acid in addition to one minor peak. The contents of tube No. 86 (F 86) of the main peak fraction were used for analyses of the amino acid composition

TABLE 2. *Determination of N-terminal, C-terminal and total amino acids and amino sugars in tube No. 86 in P-4 (in Fig. 5), before and after either dinitrophenylation or hydrazinolysis*

Constituent	A[a]	B[a]	C[a]
Mur	0.86	0.65	0
Glu	1.00	1.00	0
Ala	1.89	1.92	1.12
A_2pm	0.90	tr[b]	0
GlcN	0.73	0.74	0

[a] A, B and C denoted that the test specimens were submitted to no treatment, dinitrophenylation and hydrazinolysis, respectively.
[b] Trace.

and N-terminal and C-terminal amino acids (Table 2). F 86 contained muramic acid, glucosamine, alanine, diaminopimelic acid and glutamic acid in a ratio of about 0.9:0.7:1.9: 0.9:1.0. All the A_2pm residues disappeared on dinitrophenylation and about half the constituent alanine, about one mole per mole of glutamic acid, was recovered as C-terminal amino acid. All the glutamic acid residues remained after treatment of the acid hydro-

lysate of fraction F 86 with L-glutamic acid decaroxylase, and about half the alanine residues were lost on treatment with D-amino acid oxidase. Mobility of DNP-A$_2$pm formed by dinitrophenylation of acid hydrolysate of F 86 on thin layer chromatography of silica gel G coincided with DNP-*meso*-A$_2$pm.

The above results indicate that the main building block of *V. parahaemolyticus* peptidoglycan is a disaccharide(GlcNAc-MurNAc)-tetrapeptide (L-Ala-D-Glu-*meso*-A$_2$pm-D-Ala) monomer. The amounts of fractions other than P-4 were not sufficient for further analysis.

2. *Preparation of another Chalaropsis enzyme digest of V. parahaemolyticus cell wall peptidoglycan*
1) Isolation of cross-linked disaccharide-peptide subunit dimers

The principal building block of *V. parahaemolyticus* peptidoglycan was a disaccharide-tetrapeptide monomer, as described above, but analyses for the terminal amino acids of undigested peptidoglycan showed that about 30% of the A$_2$pm residues were linked to neighbouring peptide subunits (Table 3). Therefore, an attempt was made to isolate a dimer of disaccharide-peptide subunits by fractionation of another batch of *Chalaropsis* enzyme digest.

For this, 250 mg of peptidoglycan prepara-

TABLE 3. *Determination of N-terminal, C-terminal and total amino acids and amino sugars of the native (not enzymatically digested) V. parahaemolyticus cell wall peptidoglycan (step 3 preparation)*

Constituent	A[a]	B[a]	C[a]
Mur	0.61	0.82	0
Glu	1.00	1.00	0
Ala	1.93	2.04	0.39
A$_2$pm	0.82	0.27	0
GlcN	0.72	0.82	0

[a] A, B and C as in Table 2.

tion at step 3 (Tamura et al., 1976) was digested with 0.5 mg of the *Chalaropsis* enzyme in 120 ml of 0.025 M acetate buffer, pH 4.5 at 37 C for 48 hr. The digest was centrifuged at 100,000 × g for 50 min to precipitate particulate polyglucose and poly-β-hydroxybutyrate and the precipitate was washed twice with water. The supernatant after separation of insoluble material, which amounted to 196 mg or 78% of the starting material, and the washing were combined and concentrated in a rotary evaporator.

The concentrated solution was applied to the top of a Sephadex G-75 column (2 × 100 cm) and eluted with water. As shown in Fig. 6, a barely discernible peak of the higher molecular weight fraction (F I, tube Nos. 11–27) and a sharp peak of the lower molecular weight fraction (F II, tube Nos. 28–36) were obtained.
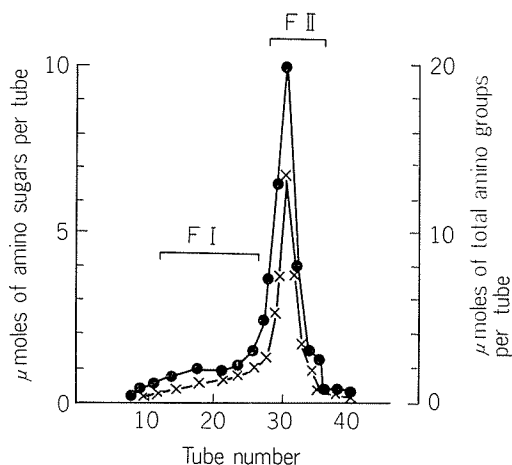

FIGURE 6. Gel filtration of the *Chalaropsis* enzyme digest of *V. parahaemolyticus* peptidoglycan on a Sephadex G-75 column. Fractions of 10 ml of eluate were collected and assayed for total amino groups (●——●) and amino sugars (×——×).

Chemical analyses (Table 4) showed that F I contained 10.1 and 30.5 μmoles of total amino sugars and amino groups, respectively, whereas F II contained 22.9 and 69.3 μmoles, respectively. The molar ratio of either total

TABLE 4. *Contents and ratio of total amino sugars and total amino groups in Sephadex fractions F I and F II of the Chalaropsis enzyme digest (Fig. 6)*

Determination	F I (μmoles)	F II (μmoles)	Ratio (F I : F II)
Total amino sugars	10.07	22.97	1 : 2.28
Total amino groups	30.50	69.29	1 : 2.27

amino sugars or amino groups in both F I and F II was approximately 1 : 2.

2) Purifications of F I and F II by column chromatography on Dowex-1

The method used for rechromatography of fraction P-4 was used. The elution profiles of total amino groups of F I and F II are shown in Figs. 7 and 8, respectively. F I separated into several peak fractions: the biggest fraction (F I-A) was eluted with around 2.5 M acetic acid and another main fraction (F I-B) was eluted with 3 M acetic acid.

Analytical data on F I-A and -B, summarized in Table 5, show that both fractions contained approximately equimolar amounts of muramic acid, glucosamine, glutamic acid and A_2pm and twice this much alanine (the muramic acid residues were probably destroyed during acid hydrolysis). Terminal amino acid analyses revealed that about half the A_2pm residues remained after dinitrophenylation and 0.2 to 0.3 mole per mole of D- or L-alanine residues were released by hydrazinolysis.
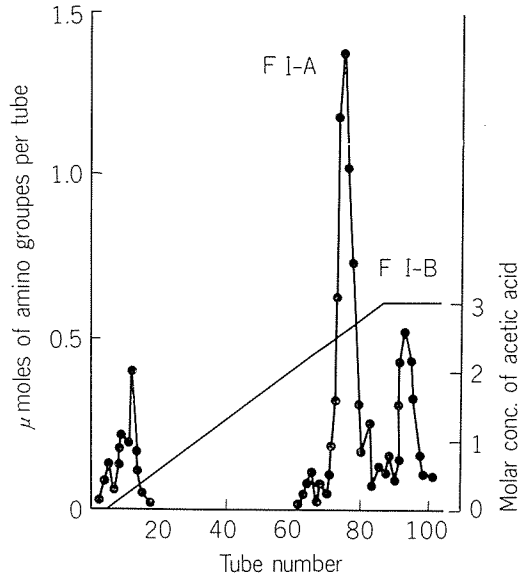

FIGURE 7. Column chromatography of the Sephadex fraction (F I in Fig. 6) on a Dowex-1×8 (acetate form). Fractions of 5 ml of eluate were collected and assayed for total amino groups (●——●).

Thus both F I-A and B were bisdisaccharide-tetrapeptide dimers, and they were probably separated from each other on the Dowex column by differences in the degrees of amidation of their glutamic acid or A_2pm residues, and consequently by differences in electric charge.

Analyses (Table 6) of the total, N-terminal and C-terminal amino acids of the principal peak fraction (eluted with 2 M acetic acid) ob-

TABLE 5. *Determination of N-terminal, C-terminal and total amino acids of F I-A and B (tube Nos. 71–79 and 91–98 in Fig. 7), before and after either dinitrophenylation or hydrazinolysis*

Constituent	F I-A			F I-B		
	A[a]	B[a]	C[a]	A[a]	B[a]	C[a]
Mur	0.32	0.26	0	0.68	0.57	0
Glu	1.00	1.00	0	1.00	1.00	0
Ala	2.00	2.45	0.27	2.05	2.03	0.21
A_2pm	0.83	0.51	0	0.84	0.66	0
GlcN	0.85	0.80	0	0.83	0.79	0

[a] A, B and C as in Table 2.

TABLE 6. *Determination on N-terminal, C-terminal and total amino acids and amino sugars of tube Nos. 90–100 fraction (Fig. 8), before and after either dinitrophenylation or hydrazinolysis*

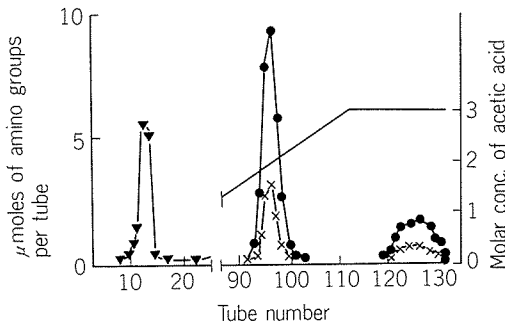| Constituent	A[a]	B[a]	C[a]
Mur	0.76	0.68	0
Glu	1.00	1.00	0
Ala	2.04	1.97	0.49
A_2pm	0.99	tr[b]	0
GlcN	0.86	0.86	0

[a] A, B and C as in Table 2.
[b] Trace.

FIGURE 8. Column chromatography of the Sephadex fraction (F II in Fig. 6) on a Dowex-1 × 8 (acetate form). Fractions of 10 ml of eluate were collected and assayed for total amino groups (●——●), amino sugars (×——×) and reducing sugars (▼——▼).

tained by Dowex column chromatography of F II (Fig. 8), indicated that F II was mainly composed of a disaccharide-tetrapeptide monomer.

3. Determination of the chain length of the glycan moiety of V. parahaemolyticus cell wall peptidoglycan

An L-11 enzyme digest was used in place of the *Charalopsis* enzyme digest of *V. parahaemolyticus* peptidoglycan. A sample (505 mg) of the peptidoglycan preparation (step 3) was incubated with 50.5 mg of the crude L-11 enzyme (lot No. SE 64) in 50 ml of 0.01 M

veronal buffer, pH 8.5 at 37 C for 72 hr. During the incubation, the free amino groups in the mixture increased from 0.59 mole per mole of total glutamic acid residues (222.9 nmoles per mg) at zero time to 1.28 nmole after 72 hr. The whole lysate was centrifuged at $100,000 \times g$ for 30 min and the supernatant was removed and concentrated in a rotary evaporator. Then it was applied to a Sephadex G-75 column (2.2 × 100 cm) and the column was eluted with water. The free amino groups, total amino sugars, reducing power and hexoses in each fraction was determind.

The elution profile is shown in Fig. 9. Samples equivalent to 200 nmoles of amino sugars of the nine fractions obtained were used for determination of the glycan chain length and for analyses of amino sugars at the reducing end (Table 7).

The test fraction with either glucosamine or muramic acid at the reducing end had chain lengths of 2 to 44 hexosamine units. The
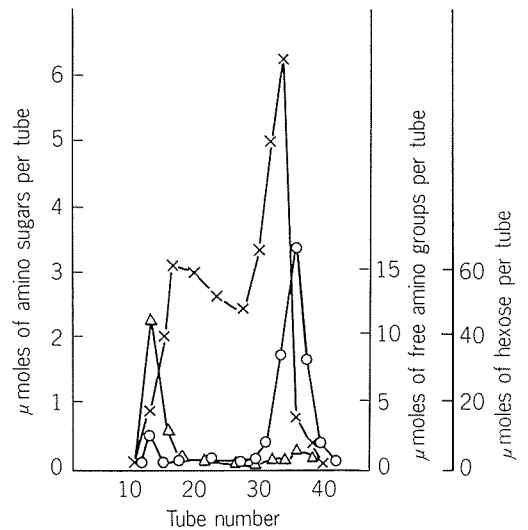
FIGURE 9. Gel filtration of the L-11 enzyme digest of *V. parahaemolyticus* cell wall peptidoglycan on a Sephadex G-75 column. Fractions of 10 ml of eluate were collected and assayed for free amino groups (○——○), amino sugars (×——×) and hexose (△——△).

TABLE 7. *Distribution of glycans with different chain lengths isolated from the L-11 enzyme digest of V. parahaemolyticus cell wall peptidoglycan on a Sephadex G-75 column*

Tube No.	Amino sugar at reducing end	Chain length[a]	Percentage of each fraction in terms of total amino sugar[b]
14	GlcN, Mur	8, 7	9
16	GlcN, Mur	35, 14	14
20	Mur	28	12
25	Mur	44	11
30	Mur	43	22
32	Mur	8	28
33	Mur	3	4
34	Mur	2	2
36	Mur	2	2

[a] Chain length $= \dfrac{\text{glucosamine} + \text{glucosaminitol}}{\text{glucosaminitol}} \times 2$ or $\dfrac{\text{muramic acid} + \text{muramicitol}}{\text{muramicitol}} \times 2$

[b] Percentage of each fraction to sum of all the fractions in terms of total amino sugar.

material in tube Nos. 16 to 30, which was virtually free of hexose and peptides but which contained 65% of the amino sugars of the L-11 enzyme digest of *V. parahaemolyticus* peptidoglycan, was found to have a glycan chain length of 28 to 35 amino sugar residues. The higher molecular weight fraction (tube Nos. 10–15) was unexpectedly shown to have a short chain length. The fraction contained a significant amount of hexose polymer associated with the peptidoglycan and glucosamine other than muramic acid at the reducing

end. The glycan fragments with glucosamine at reducing end were only found in the higher molecular weight fractions.

A possible molecular structure for *V. parahaemolyticus* cell wall peptidoglycan deduced from the present analytical results is shown in Fig. 10.

4. *Utilization of UDP-MurNAc-pentapeptide and the effect of penicillin G on peptidoglycan synthesis in V. parahaemolyticus and Pseudomonas sp.*

The particulate fraction prepared from *V. parahaemolyticus* A55, which was probably derived from the cytoplasmic membranes, was incubated with UDP-MurNAc-pentapeptide (labeled with ^{14}C-D-alanine) and UDP-GlcNAc for 2 hr and the incorporations of radioactivity into both the peptidoglycan and lipid intermediate were studied (Table 8). The amount of ^{14}C-alanine incorporated into the peptidoglycan was almost equivalent to the amount of ^{14}C-alanine liberated. Thus the terminal cross-linking reaction in cell wall peptidoglycan synthesis was catalyzed by a transpeptidase present in the particulate fraction from *V. parahaemolyticus*, like that in

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-GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc-
            |                |                |
          L-Ala            L-Ala            L-Ala
            |                |                |
          D-Glu            D-Glu            D-Glu
            |                |                |
       meso-A₂pm        meso-A₂pm        NH-A₂pm
            |                |               /
          D-Ala         D-Ala-CO           D-Ala
           (4)                    (1)
```
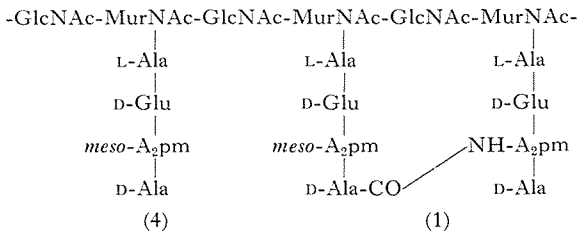
FIGURE 10. Proposed chemical structure of *V. parahaemolyticus* cell wall peptidoglycan. In this figure cross linking is shown between two of six tetrapeptide subunits on the same glycan chain. The interpeptide cross links could of course be found between the tetrapeptide subunits of different but neighbouring glycan chains. Numbers in parentheses indicate the ratio of tetrapeptide monomers to tetrapeptide dimers.

TABLE 8. *Utilization of UDP-MurNAc-pentapeptide for peptidoglycan synthesis and effect of penicillin G in V. parahaemolyticus A55 and Pseudomonas sp. 101[a]*

Strain	Penicil-lin G	Peptido-glycan product[b] (cpm)	D-Alanine released (cpm)	Lipid-inter-mediate (cpm)	Substrate not utilized (cpm)	Total (cpm)
V. parahaemolyticus A55	−	16,465	16,959	2,251	52,768	97,443
	+	22,194	13,438	2,634	63,797	102,063
Pseudomonas sp. 101	−	5,149	9,779	808	79,057	94,793
	+	9,713	3,975	809	89,491	103,988

[a] Assays were carried out as described in the methods, with or without addition of penicillin G.
[b] Sum of the peptidoglycan products which remained at the origin on paper chromatogram and materials believed to be degradation products of the peptidoglycan by an endogenous endo-N-acetylmuramidase.

Escherichia coli (Izaki et al., 1966, 1968) and *Pseudomonas aeruginosa* (Suginaka et al., 1974).

Addition of penicillin G (200 μg/ml) to the system for peptidoglycan synthesis inhibited alanine release and increased incorporation of D-alanine into the peptidoglycan, and the latter gave a broad spot on radioautograms of the products, as reported by Izaki et al. (Izaki, et al., 1966; 1968). This suggested that the transpeptidase activity of *V. parahaemolyticus* particulate fraction was partially inhibited by penicillin, and consequently that formation of uncross-linked peptidoglycan increased in the presence of penicillin.

Formation of peptidoglycan and release of D-alanine from UDP-MurNAc-pentapeptide were also observed with a particulate fraction from *Pseudomonas* sp. 101 which is more halophilic than *V. parahaemolyticus*, and the reaction was also inhibited by penicillin G.

DISCUSSION

A previous paper (Tamura et al., 1976) reported that the envelope fraction of *V. para-haemolyticus* A55 isolated by treatment of whole cells with SDS and enzymic digestion still retained a sacculus shape. Chemical analyses of a " step 3 " preparation of the cell envelope fraction which had not yet been submitted to chloroform extraction or isoamylase

digestion, showed that it consisted of 35% amino sugars and amino acids, 66% poly α-glucan and 11% poly-β-hydroxybutyrate. The main constitutive amino acids and amino sugars were alanine, glutamic acid, 2,6-di-aminopimelic acid, N-acetylglucosamine and N-acetylmuramic acid, and like those of cell wall peptidoglycan of non-halophilic gram-negative rod-shaped bacteria. However, the yield of peptidoglycan was 1.1% and as discussed in a previous paper (Tamura, et al., 1976), it seemed to be definitely lower than that of the comparable envelope fractions from *E. coli* (White, et al., 1968) and *Spirillum serpens* (Kolenbrander and Ensign, 1968).

The *Charalopsis* enzyme is an endo-N-acetylmuramidase with no endopeptidase or amidase activity and it has been used for isolation of intact peptide subunits of bacterial cell wall peptidoglycan (Tipper et al., 1964; Hash and Rothlauf, 1967). In this study, therefore, building blocks in which the peptide portion are native or intact were isolated from the *Chalaropsis* enzyme digest of *V. parahae-molyticus* cell wall peptidoglycan.

Gel filtration on serial column of Sephadex G-50, G-50 and G-25 of the *Charalopsis* enzyme digest of the cell envelope fraction resulted in good separation of polyglucose and soluble peptidoglycans. On large scale digestion of the *V. parahaemolyticus* cell envelope fraction with the *Chalaropsis* enzyme and frac-

tionation of the digest on a Sepharose or Sephadex column, it was difficult to separate the solubilized peptidoglycans from poly α-glucose, but good separation was finally achieved by repeated gel filtration: when the digest had been centrifuged at $100,000 \times g$ for 1 hr to remove poly α-glucose and poly-β-hydroxy-butyrate, disaccharide-peptides were separated well on Sephadex G-75 column, as illustrated in Fig. 6. Most of the solubilized peptidoglycan in the *Chalaropsis* enzyme digest were uncross-linked, disaccharide-peptide monomers and from the elution profile on a Sephadex column the ratio of these to a minor fraction was about 2 to 1 (Fig. 6). A cross-linked peptide dimer was isolated from the minor fraction of higher molecular weight by gel filtration on a Sephadex G-75 column (Fig. 6). The above ratio is consistent with the fact that 22% of the *V. parahaemolyticus* cell wall peptidoglycan was solubilized by the *Chalaropsis* enzyme and 63% of the total amino acids and amino sugars contained in the starting material were recovered in the digest.

When the native cell wall peptidoglycan fraction at " step 3 " was treated with 2,4-dinitrofluorobenzene, about 30% of the A_2pm residues were not dinitrophenylated. This indicates that roughly 30% of the A_2pm residues are in cross-links. This value of cross linking is very similar to those reported for the cell wall peptidoglycan of *Proteus mirabilis* (Katz and Martin, 1970), *Vibrio fetus* (Winter et al., 1971) and *P. aeruginosa* (Heilman, 1972), but is definitely lower than the value of 50% reported for the peptidoglycans of *E. coli* (Takebe, 1965) and a marine Pseudomonad (Forsberg et al., 1972).

The chain length of the glycan portion of *V. parahaemolyticus* peptidoglycan was measured using the L-11 enzyme digest described

in the methods and by Krulwich et al. (1967). When *V. parahaemolyticus* cell wall peptidoglycan was digested by the L-11 enzyme (Kato et al., 1968; Kato and Strominger, 1968), the net increase of free amino groups determined by the DNP method amounted to 0.69 mole per mole of total glutamic acid residues. About 70% of the amide linkages between N-acetylmuramic acid and L-alanine were probably hydrolyzed by the L-11 N-acetyl-muramyl-L-alanine amidase, because this enzyme had no significant effect on direct -D-alanyl-*meso*-A_2pm cross linkages in *V. parahaemolyticus* peptidoglycan.

The analytical results on the chain length and reducing end of the glycan portion obtained using the L-11 enzyme digest are summarized in Table 7. The glycans with muramic acid at the reducing end and a chain length of 28 to 44 contained about 45% of the total amino sugars in the fraction. The overall chain length varied from 7 to 44, and a small amount of solubilized glycan recovered in the higher molecular weight fraction was found to have glucosamine at the reducing end.

The particulate fractions obtained by differential centrifugation of the sonicated preparation of *V. parahaemolyticus* using the halophilic *Pseudomonas* sp. 101 as a reference were shown to catalyze penicillin-sensitive transpeptidation between UDP-MurNAc-[14]C-pentapeptide and UDP-GlcNAc in a similar way to that reported for the particulate enzymes of *E. coli* (Izaki et al., 1966; 1968) and *P. aeruginosa* (Suginaka et al., 1974). Thus in this work the existence of interpeptide subunit cross bridges in the wall peptidoglycan of halophilic *V. parahaemolyticus* was demonstrated by biosynthesis, but no quantitative information was obtained on formation the interpeptide cross links.

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