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Confirmation and complete ^{1}H and ^{13}C NMR assignment of the structure of peptidoglycan from *Sarcina ventriculi*, a highly adaptable Gram-positive bacterium

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Abstract

The structure of peptidoglycan extracted from the Gram-positive bacterium *Sarcina ventriculi* grown at pH 3 was characterized by amino acid analysis, mass spectrometry, and two-dimensional NMR spectroscopy. The basic muropeptide subunit consisted of an *N*-acetylglucosamine-β-1,4-*N*-acetylmuramic acid disaccharide substituted with an oligopeptide with the sequence Ala–*iso*Gln–A_2pm(–Gly)–Ala. The dimeric muropeptide was also characterized as a cross-linked bis-disaccharide-penta-hexapeptide with the structure, GlcNAc–MurNAc–Ala–*iso*Gln–A_2pm(–Gly)–Ala → GlcNAc–MurNAc–Ala–*iso*Gln–A_2pm(–Gly)–Ala–Ala. These results are consistent with a structure proposed based on enzymatic degradation and chemical modifications but with no use of spectroscopic information [O. Kandler, D. Claus, and A. Moore, *Arch. Mikrobiol.*, 82 (1972) 140–146]. The cell wall of this organism is very tightly cross-linked and is much more rigid than that of most other Gram-positive bacteria. There is, however, a large degree of conservation in the general structure compared to peptidoglycan in other bacteria that are not well adaptable to extremes, indicating that the membrane plays a more important role in adaptation. © 1997 Elsevier Science Ltd.

Keywords: Peptidoglycan; *Sarcina ventriculi*; Structure; NMR spectroscopy; Muropeptide; Adaptation

1. Introduction

Peptidoglycan is a uniquely bacterial macromolecule that forms the rigid cell wall of both Gram-negative and Gram-positive bacteria. It consists of a glycan backbone of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) of the linkage type [GlcNAcβ1 → 4MurNAc] with a short peptide chain (typically 3–5 amino acids long) linked to the lactyl moiety of muramic acid. There is cross-linking between the tetrapeptides of adjacent glycan strands usually involving the carboxyl of a C-terminal D-alanine of one peptide and the ω-amino group of a diamino acid. Peptide cross-linking bonds between amino acid residues located on different glycan chains lead to the

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formation of a complex three-dimensional macromolecule surrounding the cell [1]. Although the nature of the oligopeptides and the peptide bridge are both variable among bacterial groups [2], the general structure of a rigid arrangement of polymeric glycan cross-linked by peptides has been well conserved.

The muropeptides obtained by muramidase digestion have been isolated by reverse phase high-performance liquid chromatography (HPLC) [3,4]. Monomeric muropeptides have been characterized by fast-atom bombardment MS (FABMS) [5–8], tandem mass spectrometry (FABMS/MS) [9–11], plasma desorption MS (PDMS) [12,13], and specific enzymatic degradation [14]. The latter method has been used to support a proposed structure for the peptidoglycan of *Sarcina ventriculi* [15]. This structure has not been confirmed by spectroscopic methods.

Sarcina ventriculi is a highly adaptable Gram-positive organism that can be cultured under various conditions including pH values ranging from 3 to 10 [16] and in the presence of a wide variety of organic solvents [17]. During normal growth, the pH of the medium becomes quite depressed and can drop to below 4 [16]. The specific adaptation of plasma membrane components of *Sarcina ventriculi* cells grown at low pH has been studied and the formation of unusual transmembrane lipid species identified as a key adaptive response [17,18]. These lipids span the membrane, thus stabilizing it. Whether the peptidoglycan has any unusual structural features in cells grown under these conditions is unknown. It is known that peptidoglycan structure might be sensitive to environmental conditions [19]. Here we describe the isolation and characterization of muropeptides of *Sarcina ventriculi* from cells grown at pH 3 by a combination of mass spectrometry and NMR spectroscopy in an effort to determine whether there are any unusual modifications. This study is also a critical step in providing NMR spectroscopy data that will allow the characterization of the three-dimensional structure of the *Sarcina ventriculi* peptidoglycan by nuclear Overhauser effect measurements and angular constraints.

2. Results and discussion

The separation profile of muropeptides by size exclusion chromatography on a Bio-Gel P4 column is shown in Fig. 1. Samples from the peaks labeled I, II, III, and IV were subjected to acid hydrolysis followed by amino acid analysis. The amino acids found in all fractions were alanine, glutamic acid or glutamine, diaminopimelic acid, and glycine, but in varying proportions between peaks. GC–MS analyses of the alditol acetate derivatives revealed the occurrence of GlcNAc and traces of glucose. The ^{1}H NMR spectra of fractions I, II, III, and IV revealed essentially the same features, indicating that the fractions corresponded to muropeptides of different size. The separation was good enough to permit further analysis. The ^{1}H NMR spectrum of fraction IV (the lowest molecular size component) showed the least complexity and was expected to give the most information on the basis that it might be a monomeric subunit, although some heterogeneity was expected.

The FABMS spectrum confirmed this heterogeneity. It indicated the presence of several components in fraction IV. Ions observed at *m/z* 718, 1018, and 1089 were assigned to sodiated molecular species. The sodium ions were removed with concomitant increase in sensitivity [2,13] by treating the samples with cation-exchange resin (H^+ form). The positive-ion FABMS spectrum of muropeptides so treated showed mostly intense protonated molecular ions but still showed weak sodium adduct ions (Fig. 2), allowing the molecular weights to be determined. A signal for an isotope +1 amu higher was observed for all peaks because of partial exchange of one amide proton by deuterium when the sample was dissolved in D_2O for NMR analysis. The ion at *m/z* 718 corresponded to the sodium adduct ion from $[M + H]^+ = 696$ which appeared as a doublet at *m/z*

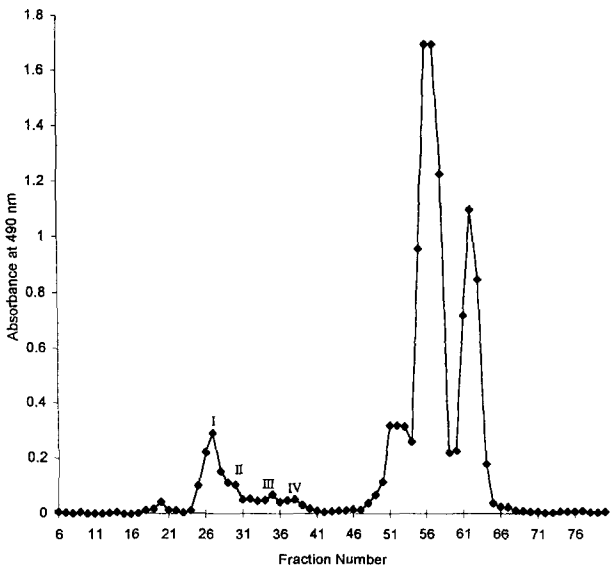

Fig. 1. Gel-filtration chromatogram on a Bio-Gel P4 column of the polar fraction of *Sarcina ventriculi*. Note that the later eluting peaks corresponded to oligosaccharides.

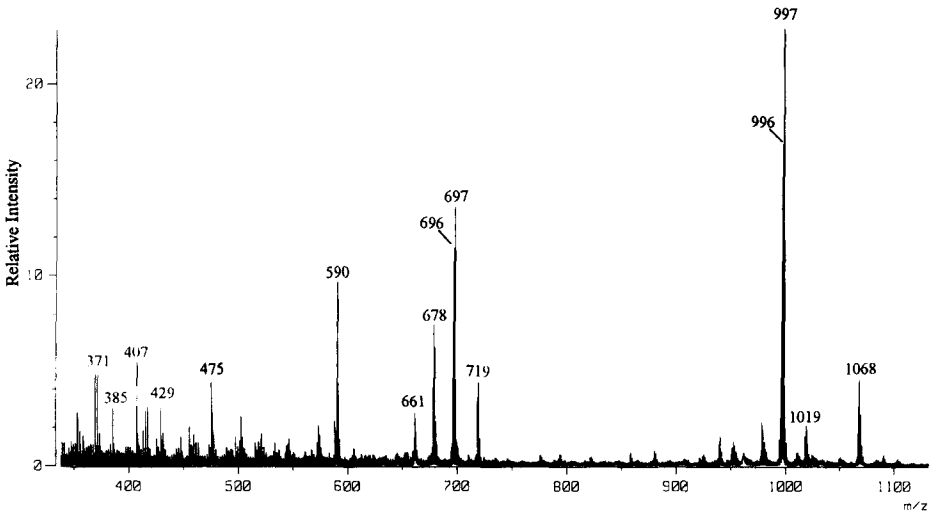

Fig. 2. Positive-ion FAB mass spectrum of the monomeric muropeptide. The complicated isotope pattern is due to the partial deuterium exchange of one amide proton because the sample was dissolved in D_2O for NMR experiments.

696/697 because of partial deuterium exchange. Based on carbohydrate and amino acid analyses, the ion at *m/z* 696 was assigned to the $[M + H]^+$ ion of the disaccharide-dipeptide: GlcNAc–MurNAc–Ala–*iso*Gln. The ion at *m/z* 678 was assigned to loss of water from the one at 696, although it could also be a protonated molecular ion of a 1,6-anhydromuramyl peptide [20]. However, this possibility was later excluded by 2-dimensional NMR spectroscopy. The ion at *m/z* 996 corresponded to the protonated ion of the muropeptide monomer GlcNAc–MurNAc–Ala–*iso*-Gln–A_2pm–Ala. The ion at *m/z* 1067 corresponded to the monomeric muropeptide containing one more alanine residue at the C-terminal. The number of carboxylic acid groups in the various molecular species were determined by FABMS after converting them to methyl esters.

To further characterize the proposed structures, the protonated molecular ion at *m/z* 997 corresponding to the most intense ion in the ion cluster was analyzed by collisionally activated dissociation tandem mass spectrometry (CAD-MS/MS). This is thought to be one of the most powerful mass spectrometry tools for the structural analysis of muropeptides [7,8]. The CAD mass spectrum yielded a large number of product ions as shown in Fig. 3. The ion at *m/z* 979 corresponded to the loss of water from the reducing end (Fig. 4). The intense ions at *m/z* 793 and 776 corresponded to the cleavage on either side of the glycosidic oxygen and the loss of GlcNAc. Monosac-

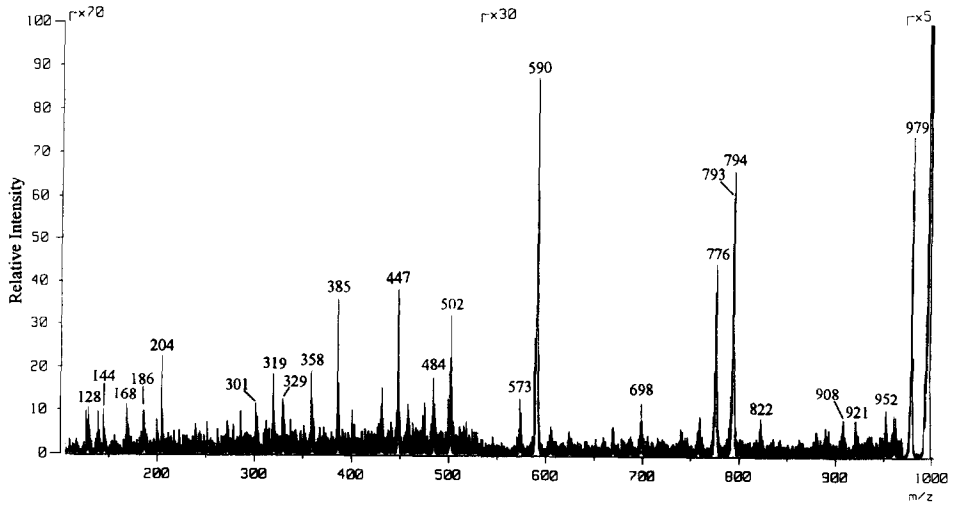

Fig. 3. Positive-ion FAB-CAD-MS/MS spectrum of GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala, $[M + H]^+$ ion at *m/z* 997.

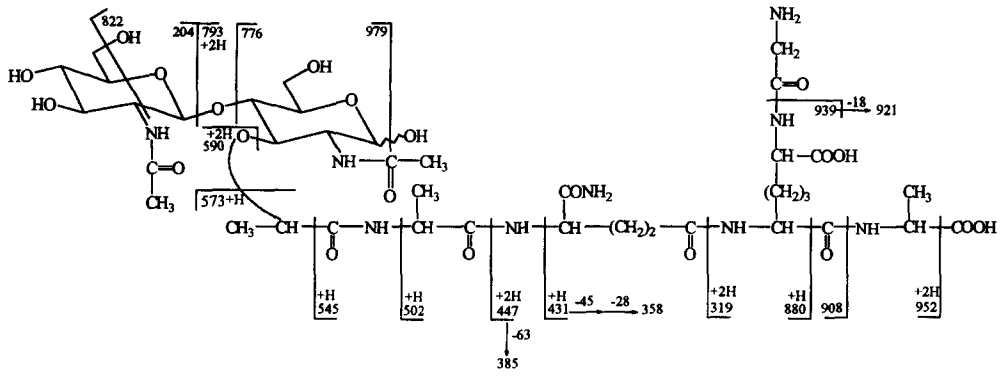

Fig. 4. Structure and fragment ions observed in the FAB-CAD-MS/MS spectrum of the $[M + H]^+$ ion at *m/z* 997.

charide-related fragment ions were observed at *m/z* 204, corresponding to the oxonium ion of the terminal GlcNAc moiety. The ion at *m/z* 590 corresponded to the subsequent loss of 203 (a second GlcNAc) from *m/z* 793. The ion at *m/z* 573 represented the loss of the disaccharide moiety (GlcNAc–MurNAc) from the $[M + H]^+$ ion. The sequence of the peptide portion could be deduced from the ions associated with the cleavage along the peptide backbone. The product ion at *m/z* 573 further dissociated to *m/z* 447 and 319 by the loss of the lactyl–Ala and *i*Gln residues. The internal fragment ions at *m/z* 358, 301, 145, and 128 corresponding to the loss of 57, 156, and 17 Da indicated the peptide sequence of *i*Gln–A_2pm–Gly. The glycine could be proposed to be linked to either the C-terminal alanine or the diaminopimelic acid. The fragment ion at *m/z* 908 indicated that the glycine was linked to the diaminopimelic acid. If the glycine residue were attached to the C-terminal alanine, the fragment ions at 922 and 895 would be observed [11].

Early eluting fraction III was analyzed by FABMS which indicated that it was a cross-linked dimer of the general structure just described. The ion at *m/z* 2045 corresponded to the cross-linked bis-disaccharide-penta-hexapeptide GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala → GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala–Ala (Fig. 5). It could be deduced that the cross bridges were formed between C-terminal alanine and glycine. The ion at *m/z* 1974 corresponded to the bis-disaccharide-penta-pentapeptide in which the terminal alanine is lacking from the species corresponding to *m/z* 2045. The ions at *m/z* 2027 and 1956 could be attributed to loss of H_2O from the molecular ions at 2045 and 1974, respectively. There was also evidence for the presence of muropeptides containing tetrasaccharide sugars due to incomplete cleavages by muramidase. Ma-

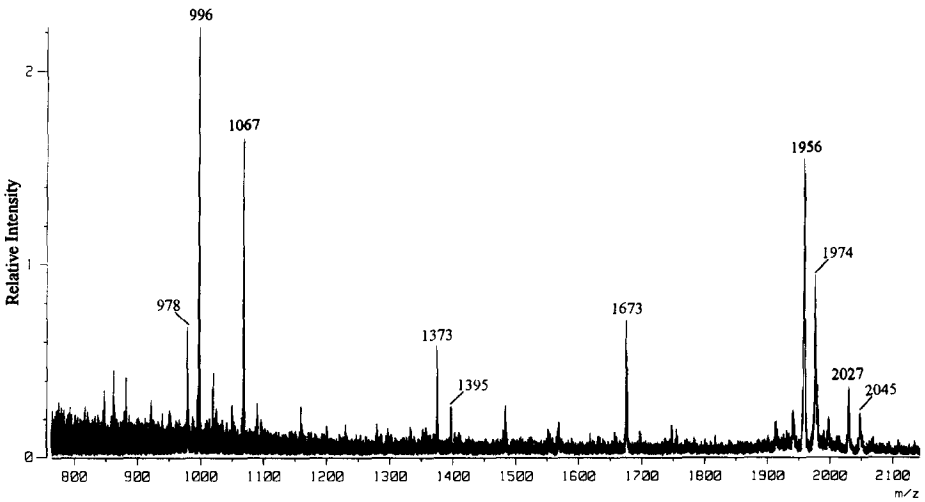

Fig. 5. Positive-ion FAB mass spectrum of fraction III.

Table 1
Structures of the muropeptides from *Sarcina ventriculi*

m/z		Primary structure
Observed	Calculated	
696	695.3	GlcNAc–MurNAc–Ala–*i*Gln
996	995.4	GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala
1067	1066.5	GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala–Ala
1974	1972.8	GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala → GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala
2045	2043.9	GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala–Ala → GlcNAc–MurNAc–Ala–*i*Gln–A_2pm(–Gly)–Ala

Table 2
^{1}H and ^{13}C chemical shifts (δ, ppm) of the disaccharide residues of the muropeptide from *Sarcina ventriculi*

^{1}H	H-1	H-2	H-3	H-4	H-5	H-6
GlcNAc	4.46	3.66	3.34	3.47	3.32	3.69, 3.86
MurNAc (α)	5.15	3.74	3.68	3.78	3.78	3.64, 3.74
MurNAc (β)	4.57	3.64	3.51	3.76	–	–
^{13}C	C-1	C-2	C-3	C-4	C-5	C-6
GlcNAc	99.9	55.6	75.6	73.1	69.8	60.6
MurNAc (α)	89.8	53.1	75.9	74.7	70.6	59.3
MurNAc (β)	94.6	55.2	78.8	74.9	–	–

trix-assisted laser desorption ionization (MALDI) mass spectrum indicated the presence of dimers and trimers in fraction II. Table 1 lists the masses of the protonated molecular ions of the components detected along with their proposed structures.

In order to confirm the proposed structures, frac-

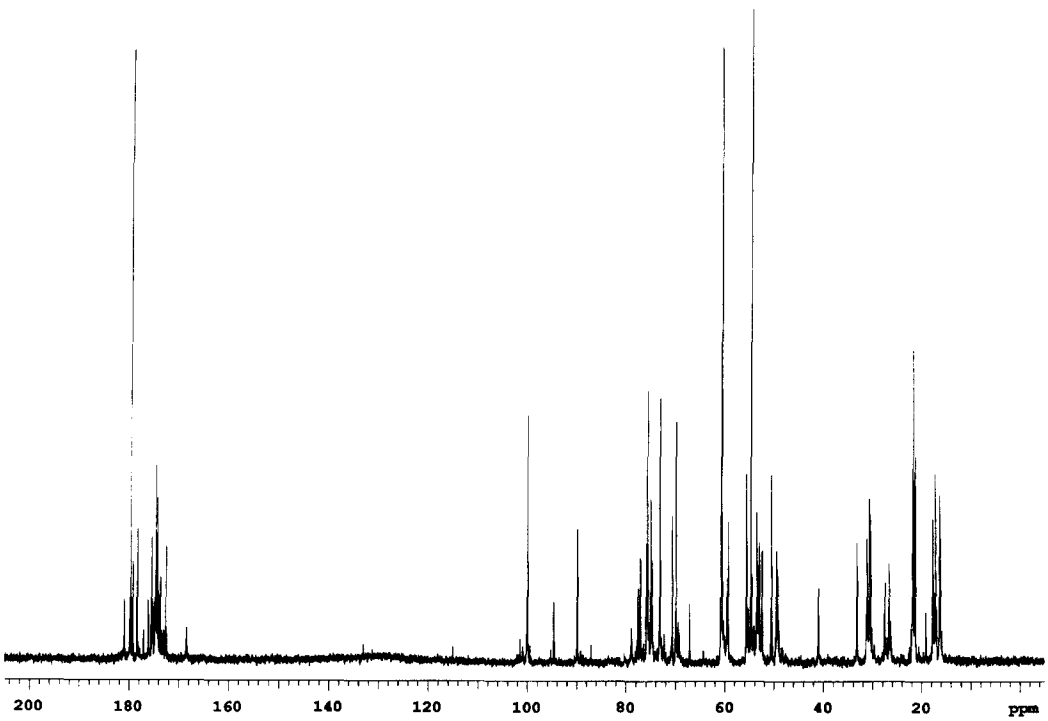

Fig. 6. ^{13}C NMR spectrum of the monomeric muropeptide.

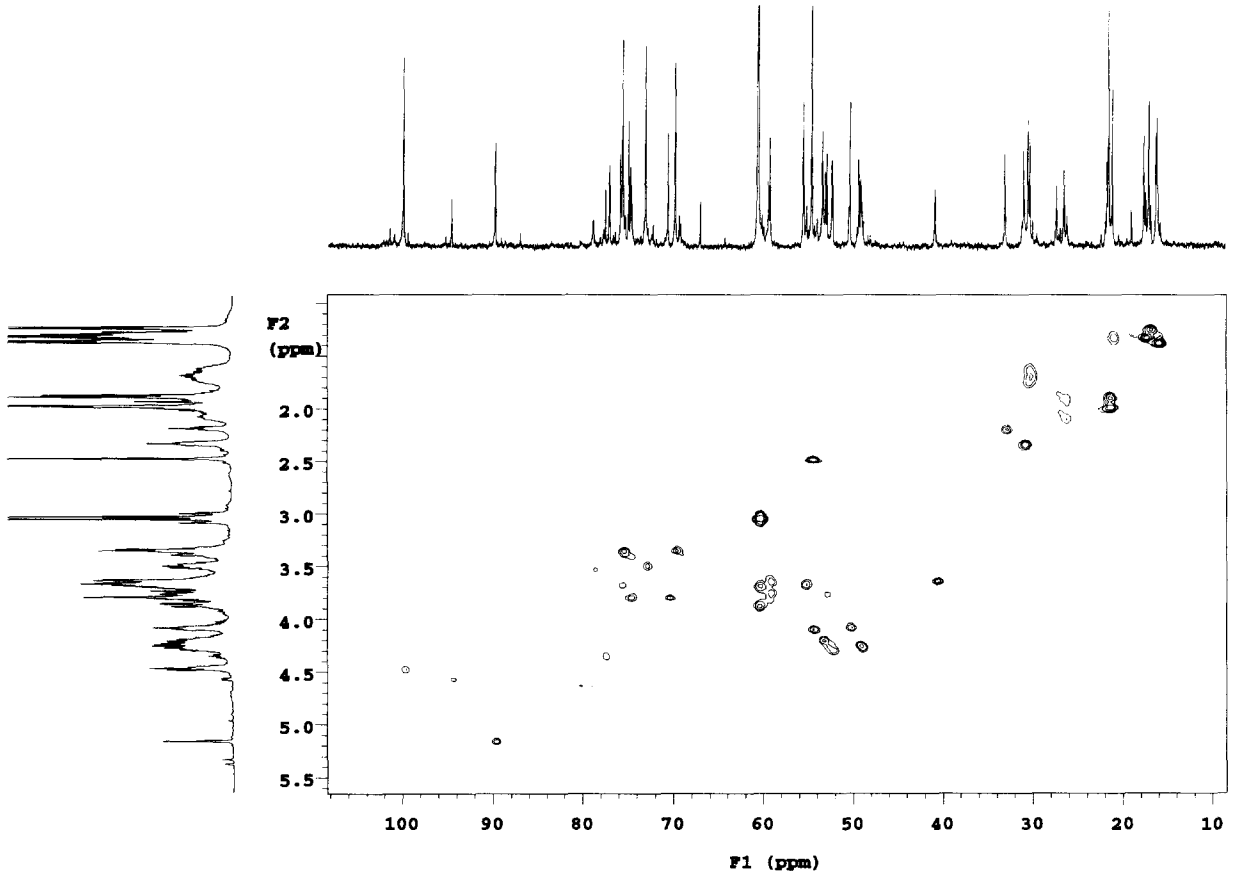

Fig. 7. $^{1}H-^{13}C$ HMQC spectrum of the monomeric muropeptide. The ^{1}H signals at 2.48 and 3.03 ppm are due to impurities.

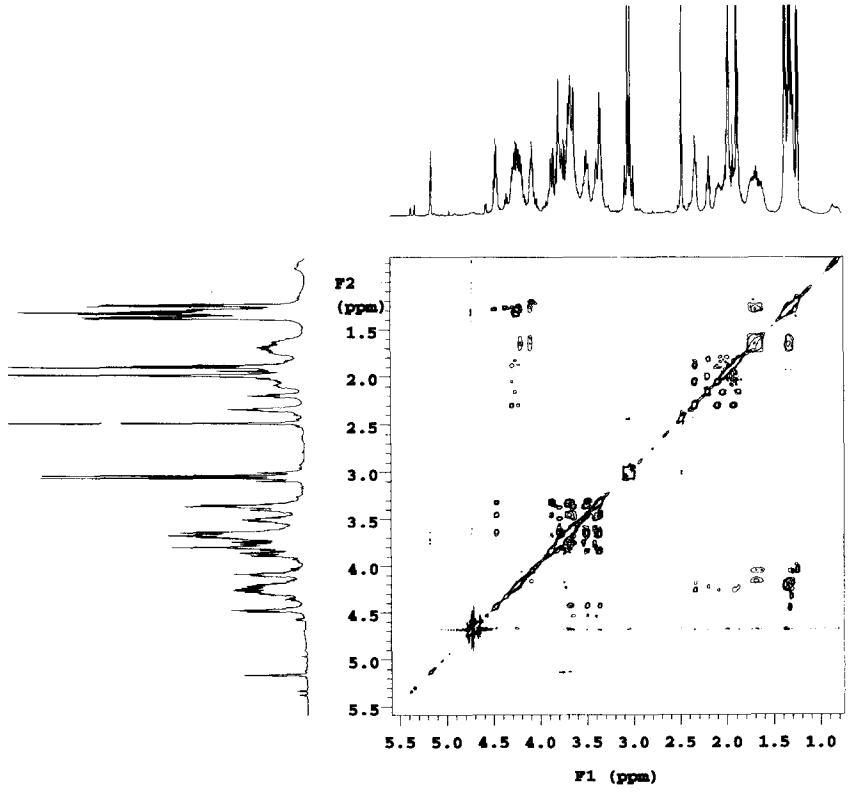

Fig. 8. HOHAHA spectrum of the monomeric muropeptide.

tion IV was analyzed by two-dimensional NMR spectroscopy. 2D NMR spectroscopy allowed the assignments of all proton and carbon resonances unambiguously. The ^{13}C spectrum of fraction IV is shown in Fig. 6. The assignments of ^{1}H and ^{13}C resonances of the disaccharide moiety are presented in Table 2. The ^{1}H NMR spectrum showed one α- and two β-anomeric signals at 5.15, 4.57, and 4.46 ppm, respectively. The doublet at 4.46 ppm (J 8 Hz) was correlated with the ^{13}C signal at 99.9 ppm in the heteronuclear multiple quantum coherence (HMQC) spectrum in Fig. 7 and was attributed to a β-anomeric carbon of GlcNAc. The $^{1}H-^{13}C$ correlated pair of signals at 4.57 (J 8 Hz) and 94.6 ppm was assigned to the β-anomeric position of MurNAc acid. The $^{1}H-^{13}C$ correlated pair of signals at 5.15 and 89.8 ppm was assigned to the α-anomeric position of MurNAc. The reason for two MurNAc signals was due to the α and β forms since this residue is at the reducing end. The proton signals belonging to one continuous spin system were traced from the proton homonuclear Hartman–Hahn (HOHAHA) spectrum (Fig. 8). The HOHAHA traces for H-1 (4.46 ppm) of GlcNAc residue showed connectivities with three signals at 3.34, 3.47, and 3.66 ppm. The signal at 3.66 ppm was coupled to the H-1 signal in the gradient-enhanced double quantum filtered J-correlated (ge-DQF-COSY) spectrum (Fig. 9). This signal was also correlated with the ^{13}C signal at 55.6 ppm and was therefore assigned to H-2 of GlcNAc. The H-2 proton signal of GlcNAc was also correlated with the signal at 3.34 ppm. The latter signal at 3.34 ppm was, thus, assigned to the H-3 proton. The resonances for H-5 (3.32 ppm) was very close to the H-3 signal and was coupled to one of the H-6 signals at 3.86 ppm. The H-6 signals appeared as two doublet of doublets at 3.86 and 3.69 ppm. The H-6 proton signals were correlated with the ^{13}C signals at 60.6 ppm. The α-anomeric proton of MurNAc showed three cross peaks at 3.68, 3.74, and 3.76 ppm in the HOHAHA spectrum. These three signals were assigned to H-3, H-2, and H-4, respectively. The H-5 and H-6 resonances were assigned from the HMQC spectrum. The assignments were verified by a heteronuclear multiple-bond correlation (HMBC) experiment showing two- and three-bond coupled cross peaks (data not shown). If MurNAc had contained a 1,6-anhydro linkage, there would have been another

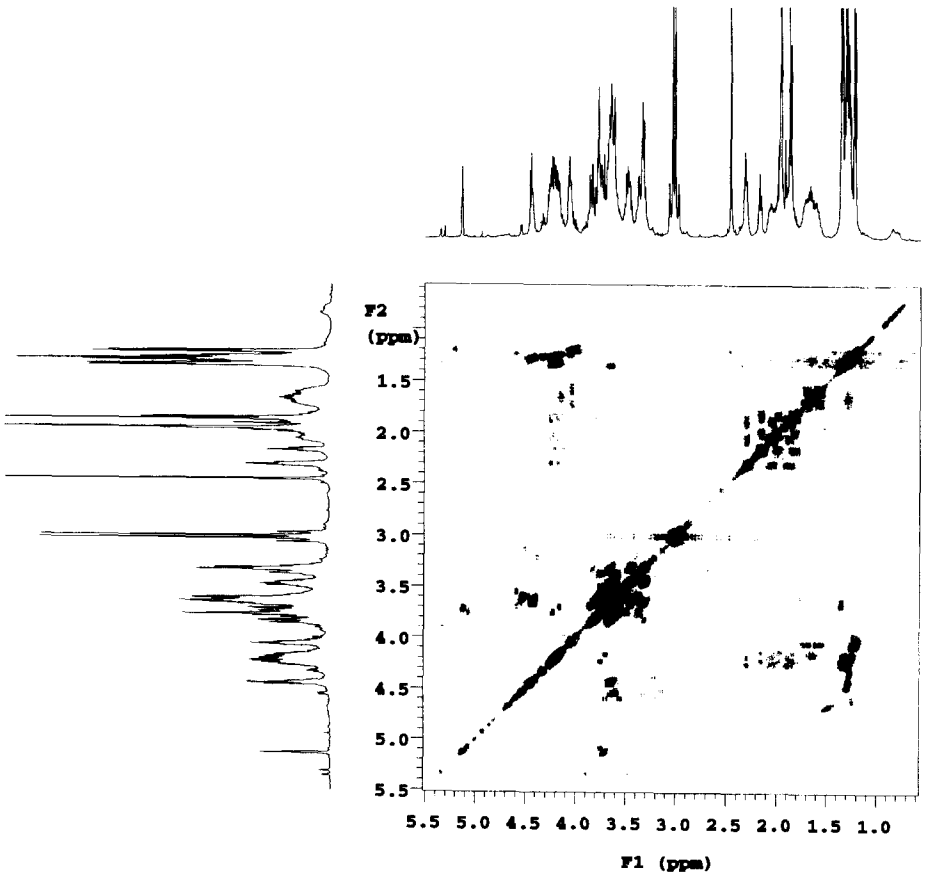

Fig. 9. ge-DQF-COSY spectrum of the monomeric muropeptide.

C-6 signal downfield of these C-6 signals. There was no indication of additional downfield C-6 signals. Thus, the ions at 678 and 978 in the FAB mass spectrum were due to the fragment ions instead of molecular ions from 1,6-anhydro muramyl compounds. The HOHAHA trace of the β-anomeric signal of MurNAc showed four cross peaks at 3.39, 3.51 (H-3), 3.64 (H-2), and 3.76 (H-4) ppm. The ge-DQF-COSY spectrum indicated that the signal at 3.39 ppm did not belong to the α-MurNAc spin system. Three singlets at 1.87, 1.89, and 1.97 ppm were assigned to the protons of *N*-acetyl groups of GlcNAc and α/β MurNAc residues, respectively. The signal at 1.31 ppm was correlated with the ^{13}C signal at 17.7 and 19.1 ppm. These methyl signals were assigned to the methyl signal of the lactic group from the MurNAc residue. The methyl signals of the lactic groups were coupled to the signals at 4.45 and 4.35 ppm in the HOHAHA and ge-DQF-COSY spectra. Thus, these latter signals were due to the methine signals of the lactic acid group. These methine proton signals showed cross peaks at 77.0 and 77.5 ppm in the HMQC spectrum and, as expected, were correlated with the carbonyl carbon of the lactic acid group at 175.4 ppm in the HMBC spectrum.

The assignments of ^{1}H and ^{13}C signals of the amino acid residues are presented in Table 3. The proton signals at 1.24 and 1.36 ppm were assigned to the H-β of alanine. In the HMQC spectrum, these methyl signals at 1.24 and 1.36 ppm were correlated with the ^{13}C signals at 17.2 and 16.3 ppm, respectively. These H-β of Ala were correlated with H-α at 4.06 and 4.25 ppm. The carbonyl signals at 174.5 and 179.2 ppm were assigned from the cross peaks between the H-α and carbonyl carbons in the HMBC spectrum. The signal at 179.2 ppm was assigned to the C-terminal Ala residue. The triplet signals at 2.18 and 2.33 ppm were assigned to the H-γ of the *iso*-glutamine residue. The triplet at 2.18 ppm was coupled to the signals at 1.92 and 4.23 ppm. The triplet at 2.33 ppm was correlated with the signals at 1.99 and 4.28 ppm in both HOHAHA and ge-DQF-COSY spectra. These multiplet signals at 1.92 and 1.99 ppm appeared underneath the methyl signals from *N*-acetyl groups corresponding to the H-β signals of the *i*Gln residues. The signals at 4.23 and 4.28 ppm were assigned to the H-α of the *i*Gln residue. The H-γ signals at 2.18 and 2.33 ppm showed cross peaks with the carbonyl carbons at 180.8 and 174.4 ppm in the HMBC spectrum. The signal at 180.8 was assigned to the C-terminal carboxyl group from the GlcNAc–MurNAc–Ala–*i*Gln molecule. The presence of diaminopimelic acid was further confirmed from the NMR spectra. The proton signal at 1.29 ppm was correlated with the ^{13}C signal at 21.3 ppm in the HMQC spectrum. The carbon chemical shift indicated that this signal was due to a methylene signal. This methylene signal was assigned to the H-γ of the A_2pm. The H-γ signal was correlated with the signals at 1.68, 4.08 and 4.17 ppm in the HOHAHA and ge-DQF-COSY spectra. The complex multiplet at 1.68 ppm which was correlated with the ^{13}C signal at 30.4 and 30.6 ppm in the HMQC spectrum corresponded to the H-β of the A_2pm. The signals at 4.08 and 4.17 ppm were assigned to the H-α. Two sets of the H-α and H-β signals appeared with different chemical shifts due to the different chemical environments of the two terminals of the diaminopimelic acid residue.

The peptide unit of peptidoglycan usually consists of four amino acids, with the general sequence Ala–Glu–X–Ala, where X is usually lysine or di-

Table 3
^{1}H and ^{13}C chemical shifts (δ, ppm) of the peptide residues of the muropeptide from *Sarcina ventriculi* [a]

^{1}H	H-α	H-β	H-γ	
Gly	3.63			
Ala	4.06/4.25	1.24/1.36		
*i*Gln	4.28/4.23	1.84, 2.01/1.90, 2.08	2.18/2.33	
A_2pm	4.08/4.17	1.68	1.29	
^{13}C	C-α	C-β	C-γ	Carbonyl
Gly	41.0			174.2
Ala	49.3/50.4	16.3/17.2		174.5/179.2
*i*Gln	52.4/53.0	26.6/27.5	31.1/33.2	180.8/174.4
A_2pm	53.5/54.7	30.4/30.6	21.3	172.5/178.3

[a] More than one entry is given for some signals because of heterogeneity, leading to different chemical shifts for the some nuclei in different environments. The muropeptide fraction IV contained two minor components in addition to the major one.

aminopimelic acid. The glutamyl linkage is always in the γ position and the α-carboxyl of the glutamic acid may either remain unsubstituted, be amidated (as in *Staphylococcus aureus*) or aminoacylated (as in *Micrococcus luteus*) [21]. The peptidoglycan monomer of *Sarcina ventriculi* described earlier [15] and verified here belongs to the group A3γ according to Kandler classification [22]. This type of cross-linkage is also observed in *Propionibacteria*, some *Arthobacter*, *Clostridia*, and *Streptomyces* [22]. The very short interpeptide bridge observed in *Sarcina ventriculi* should result in a more tightly cross-linked structure. However, the general structural conservation of peptidoglycan in *Sarcina ventriculi* is still remarkable and indicates that the adaptation process to very extreme environments occurs largely at the level of plasma membrane chemistry [17,23,24].

The three-dimensional structure of peptidoglycans is still not known. The three-dimensional structure of peptidoglycan from *Sarcina ventriculi* is especially important to us because of ongoing efforts to define the chemistry of the cell wall of this organism in extreme in detail in order to understand its organization and the structural modifications it undergoes during bacterial adaptation. The NMR spectroscopy assignments will make the calculation of the three-dimensional structure a real possibility.

3. Experimental

Isolation and purification of muropeptides.—*Sarcina ventriculi* was cultured as previously described [14]. Cells were extracted by treating a suspension in a buffer consisting of 25 mM EDTA and 50 mM Tris–HCl (1:1) with lysozyme (50,000 units) and protease (7 units) at 37 °C for 5 h. The slurry was then extracted with *n*-propanol. The propanol–water soln was concd to dryness and chromatographed on a C18 column (30 × 2.5 cm) which was eluted sequentially with water, water–MeOH (1:1), MeOH, MeOH–$CHCl_3$ (1:1), and $CHCl_3$ as eluents. The aq fractions were pooled and chromatographed on a Bio-Gel P4 column (200 × 2 cm) using water as the eluent. Fractions of 6 mL were collected. These were assayed for carbohydrate by the phenol sulfuric acid method [25]. The early eluting fractions belonging to the same peak were pooled.

Mass spectrometry.—The fractions were pooled and analyzed by FAB mass spectrometry with an instrument setting for a mass range from *m/z* 0 to 2250. FAB mass spectra were recorded using a JEOL HX-110 double-focusing mass spectrometer with a dithiothreitol (DTT) and thioglycerol (TG) mixture in the ratio of 1:2 as a matrix [26]. The accelerating voltage was 10 kV and the resolution was set at 1000. The desalting of the samples was done using cation-exchange resin (Bio-Rad AG 50W-X8). For this, several particles of resin were added into the aq samples containing 2% CH_3COOH. The suspension was then vortexed for several minutes. In order to determine the number of free carboxyl groups, the sample was methylated with excess diazomethane in ether for 20 min at room temperature. The CAD-MS/MS was conducted by scanning the electric sector and magnetic sector in a fixed ratio (B/E linked scan) [27]. Helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the parent ion by 50%. MALDI mass spectrometry was performed in the positive-ion mode on a PerSeptive Biosystems Voyager Elite laser desorption time-of-flight instrument with a nitrogen UV laser. 2,5-Dihydroxybenzoic acid was used as matrix. For carbohydrate analysis, the sample was hydrolyzed with 2 M CH_3COOH, reduced with $NaBH_4$, and then acetylated with Ac_2O and pyridine. GC–MS analysis of the alditol acetates was performed on a Hewlett–Packard 5995C GC–MS, equipped with a Supelco DB-225 fused-silica capillary column using a temperature program of 170 °C (3 min)–230 °C at 2 °C/min. Helium was used as the carrier gas.

Amino acid analysis.—Aliquots of muropeptides were hydrolyzed in 6N HCl (24 h, 100 °C), derivatized with phenylisothiocyanate, and subjected to amino acid analysis by HPLC (Waters) using a C18 column (3.8 × 250 mm) and a Waters 440 UV detector (λ = 254 nm).

NMR spectroscopy.—All NMR spectra were measured in D_2O at 500 MHz for ^1H or 125 MHz for ^{13}C with a Varian VXR 500 spectrometer. For the HMQC experiments [28], a spectral width of 23,202 Hz was employed for the ^{13}C dimension. A total of 64 transients were acquired at 1024 points each. A total of 512 data sets were acquired. The ge-DQF-COSY (phase sensitive mode) spectra were obtained using a total of 512 data sets (32 transients at 2048 data points each). The HOHAHA experiments [29] were performed using a total of 1024 data sets with 64 transients at 2048 data points each. A mixing time of 80 ms was used. The HMBC experiments were carried out with a spectral width of 23,202 Hz for the ^{13}C dimension. A total of 256 data sets were acquired with 32 scans and 1024 data points each.

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